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To the Graduate Council:

I am submitting herewith a thesis written by Katherine Jones Irwin entitled "The effect of nutritionally adequate ketogenic diets consistent in protein, fiber and micronutrient content on seizures in EL/Suz mice." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Nutrition.

Melissa Hansen-Petrik, Major Professor

We have read this thesis and recommend its acceptance:

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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Jung Han Kim

Lisa Jahns

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The effect of nutritionally adequate ketogenic diets consistent in protein, fiber and micronutrient content on seizures in EL/Suz mice

A Thesis Presented for
the Master of Science
Degree
The University of Tennessee, Knoxville

Katherine Jones Irwin
May 2009

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ACKNOWLEDGEMENTS

This project is dedicated to my precious son, Joel Irwin, who has endured the challenges of epilepsy. Joel has taught me not to settle for "there are no answers," but to find strength to seek those answers from the One who ultimately holds them, my Lord, Jesus Christ.

Many thanks…
To my best friend and life partner, my husband, Dennis, who has never failed to cheer me on, even when it meant that he must assume the roll of father *and mother* to my children on occasions.
To my mother, Rose Jones, who has supported me and helped to keep my household running during these years.
To my children, Joel and Erica, who have kept my life in prospective; who have taught me to truly appreciate the real gifts in life.

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Last, but not least, I thank Melissa Hansen-Petrik for the mentoring and teaching that has molded my love for science and has spurred an unsettled notion that more research is needed.

ABSTRACT

The ketogenic diet (KD) is an effective mode of therapy for epilepsy, although the mechanism(s) of action remains unknown. In investigating mechanism(s) of action, controlling for dietary factors, such as protein, fiber, vitamins and minerals, is a critical component of experimental design, particularly as many nutrients modify metabolism and gene regulation. Furthermore, experimental nutrient and energy deficient diets are not readily applicable to humans. Nonetheless, a nutritionally adequate nutrient-controlled KD has not previously been developed and evaluated for utility in an experimental model. In this study, we tested the efficacy of novel ketogenic and calorie restricted diets while maintaining consistent protein, fiber and micronutrient levels across groups in a natural model of epilepsy. Female EL/Suz mice(n=20), 100-200 days old, were randomized into four groups to independently assess the effects of carbohydrate and fat modification as well as calorie restriction. Groups included control (C), calorie-restricted (CR), ketogenic (KD), and ketogenic calorie-restricted (KDCR) diets. Mice were on treatment for 4.5-5.5 weeks. Energy levels for the calorie-restricted groups were reduced to 75% of control levels. The ketogenic groups (KD and KDCR) experienced a trend toward seizure control over the non-ketogenic groups (C and CR) although the differences were not significant. Body weights decreased significantly in the CR and KDCR groups while plasma glucose levels did not differ among groups and plasma beta-hydroxybutyrate (BHB) levels increased in the KD and KDCR groups. Mouse body weight at week 4, as well as the change in body weight at week 4 from baseline, was

significantly related to seizure control. Mice that had stable weight or weight loss were more likely to experience seizure control than mice that gained weight. The results from this study suggest that ketosis indicated by elevated BHB levels and energy restriction indicated by weight loss correspond to lower seizure susceptibility in an experimental model of naturally occurring epilepsy. Nutrient-controlled experimental diets can and should be used for investigating KD mechanisms in experimental models.

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Research hypothesis and specific aims

Although the ketogenic diet was medically recognized in the 1920s as a viable treatment for intractable pediatric epilepsy, the underlying mechanism(s) is unknown. While more and more researchers have undertaken the task to explore mechanisms underlying the efficacy of the ketogenic diet, experimental diet designs in animal models have generally been poorly controlled and/or deficient in essential nutrients.

The central hypothesis is that ketosis elicited through highly controlled experimental ketogenic diets and calorie restricted diets with equivalent protein, fiber, and micronutrient intake across groups will decrease seizures in a natural mouse model of epilepsy. The proposed research will examine seizure control and metabolic parameters exerted by ketogenic diets and calorie- restricted diets in which protein, fiber and micronutrients are standardized.

The rationale for this study is that typical experimental designs that have been used in studying this diet have not included control measures for nutrient intake across groups, thus yielding results that cannot be readily interpreted. By controlling for protein, fiber and micronutrients in experimental designs, study

results can be more clearly interpreted, thus enhancing the overall knowledge of mechanisms involved in seizure control. Insights into the mechanism(s) of this proven treatment could improve the implementation of the treatment and therefore maximize the utility of the ketogenic diet in children and adults with intractable epilepsy.

To test the central hypothesis, the following specific aims are proposed:

1. **Determine the extent to which a nutrient controlled ketogenic diet exerts anti-epileptic effects in a natural mouse model of epilepsy**. Working hypothesis: A high fat, low carbohydrate ketogenic diet reduces epileptic seizures in a natural model of epilepsy when intake of protein, fiber, and micronutrients are maintained at consistent levels.

2. **Determine the extent to which a nutrient controlled calorie-restricted diet exerts anti-epileptic effects in a natural mouse model of epilepsy**. Working hypothesis: A calorie-restricted diet reduces epileptic seizures in a natural model of epilepsy when intake of protein, fiber, and micronutrients are maintained at consistent levels.

CHAPTER II
LITERATURE REVIEW

Epilepsy

Introduction

Epilepsy is a debilitating neurological disorder affecting more than 3 million Americans, many of them children (1). In the US alone, 200,000 people will receive a diagnosis of epilepsy this year (1). A majority of epilepsies are of idiopathic origin, meaning there is no known precipitating event or known defect attributed to the cause of the disorder (1, 2).

Epilepsy is characterized by the tendency to experience periods of sudden deviations in the way a person feels or acts due to an excessive electrical discharging of neurons in either a section or the entire brain. These periods of electrical discharge can range in duration from a mere few seconds to extended seizure activity such as with status epilepticus in which can only be stopped with heavy sedation such as inducing a coma in the patient. Epilepsy can include seizures of several types, including generalized seizures that involve the entire brain. Examples of these generalized events include tonic-clonic (formerly grand mal) seizures and absence seizures (formerly petit mal). Other seizure types include partial seizures that do not affect the entire brain but only part. The area of the brain affected will determine how the seizure presents (what the seizure

will look like or feel like). For example, a seizure in the motor cortex of the frontal lobe may result in involuntary movements of a hand or leg. Partial seizures can be further classified as partial simple or partial complex. The patient affected by a partial simple seizure typically remains aware of the event and can recall what took place during the event (consciousness is maintained). This seizure type is isolated to one of any sections or lobes of the brain. The complex seizure begins in the temporal or frontal lobe and affects the area of the brain that regulates awareness and alertness, thus the patient is unaware of what is happening during the seizure. The patient may have a blank stare and make meaningless movements such as climbing, drooling or lip smacking. Often, after a complex seizure, the patient is confused and tired. Partial seizures can remain in one area of the brain and resolve without affecting the remaining areas in the brain, or the electrical discharging could spread throughout the entire brain, thus evolving into a generalized seizure.

Ancient writings reveal that epilepsy is not a new disorder, most likely dating back to the beginning of the human race. The earliest writings available refer to epilepsy as the *Sacred Disease* due to the misconceptions that epilepsy was a spiritual disorder rather than physiological (3). Varying areas of thought have existed and evolved in an attempt to explain epileptic events, yet much remains unknown about this disorder. Throughout history, attempts to explain the disease have been made. One ongoing argument has been whether the origin of an epileptic event is cause by a spiritual anomaly or physical anomaly.

The earliest documentation of epilepsy is found on an ancient Babylonian cuneiform text in the Sakikku, translated as *All Diseases.* These writings were contained on 2 tablets that date between 1067 and 1046 BC (4). The writings in the Sakikku refer to epilepsy as "the falling disease" and the descriptions of the events are laced with inferences that the person with epilepsy is possessed by demonic spirits (4). One translation from an entry in these early medical writings reads

> "If epilepsy falls once upon a person [or falls many] times, [it is (as the result of) possession] by a demon or a departed spirit"(4).

Characteristics of an epileptic event such as temporary loss of consciousness, physical stiffening movements, rolling of eyes, foaming mouth and others oddities that are sometimes a part of an epileptic seizure likely contributed to this view. The aura that some patients experience before an event was considered the entering of the demon with the climax or convulsive part of the event construed as the time in which the demon took full possession. The clonic stage, or the slowing, rhythmic twitching stage, was considered the "letting go" of the demon or releasing of the victim, thus ending the event (4). This ancient writing also includes attempts to diagnose different seizure types (i.e., attribute to a certain demon) such as:

> "If the possessing demon….., and at the time of his fit he *clenches* his hands as (tightly as) though rigor (or, rigor mortis?) had seized him, and, with legs extended, is greatly convulsed; if

then the seizure abates and he begins to regain consciousness, -

hand of the e'elu-demon" (4).

In this era, nocturnal seizures were considered to be at the hand of ghosts with one account in the Sakikku translated:

"If his seizure (or, possession) always takes place in the evening,

it is the seizure of a ghost (nocturnal epilepsy)" (4).

Even with the advancement of medical science, still today the notion that epilepsy is propagated by evil spirits remains (5, 6). Several religions today continue to view epilepsy a spiritual disorder and, among these religions, ideas are diverse as to *who* or *what* is causing the anomaly. Hirsham Ismail and his team at Bradford Teaching Hospitals in the UK report that South Asian Muslims in the UK and the Indian subcontinent, contribute the seizures to spirit possession while Sikhs and Hindus attribute the events to sins committed in a former life (7). A study of teachers in urban and rural Zambia reported that 28% of teachers considered epilepsy contagious, 17.3% attributed epilepsy to spirit possession, 16.8% to witchcraft (6). This study found that these teachers were more likely to refer their students with epilepsy to a traditional healer as opposed to a physician (6).

Although mystery and "unknowns" still surround this particular disorder, modern science advancements have contributed to understanding epilepsy as physiologic in nature, thus making it better known as a medical condition as opposed to a spiritual anomaly. As early as approximately 400 BC, some were

questioning the spirituality of this disorder. Of the Hippocratic writings dating back to 400 BC and translated in 1854, the essay that addressed this disease specifically appears to introduce the thought that this disease may not involve a spiritual element, but be a physiologic disorder. Yet, to identify epilepsy as his subject, the writer(s) began with "*On the Sacred Disease*" and continued with a commonly quoted excerpt:

> "It is thus with regard to the disease called Sacred: it appears to me to be nowise more divine nor more sacred than other diseases. But has a natural cause from which it originates like other affections. Men regard its nature and cause as divine from ignorance and wonder, because it is not at all like to other diseases. And this notion of its divinity is kept up by their inability to comprehend it, and the simplicity of the mode by which it is cured, for men are freed from it by purifications and incantations" (3).

John Hughlings Jackson's work in the mid-nineteenth century made the biggest impact to bring about the current view of epilepsy being physiological in nature (8). Jackson believed epilepsy to be electrical in nature and originating in the gray matter of the brain (8). He included pathological as well as clinical observation in his studies and even went on to work with a young surgeon, Horsley, to perform the first brain resection for epilepsy on May 25, 1886 (8). Jackson's vast understanding and subsequent reporting of epilepsy has opened the doors to a greater understanding today.

With the general consensus that epilepsy is physiological in nature as opposed to spiritual, progress has been made toward the treatment. Yet even with today's knowledge through years of clinical and basic science research, much remains unknown when it comes to narrowing down etiologies of epilepsy in patients. In fact, in 70% of new diagnoses of epilepsy, the cause is unknown (1).

Prevalence

Epilepsy is a common disorder among Americans, with over 3 million diagnosed with the disorder. The Epilepsy Foundation of America reports that one out of every 10 adults will experience a seizure in their lifetime (1). They further predict that 300,000 Americans will experience their first convulsion this year, and 120,000 of them will be children. For a staggering 70% of them, the cause of the seizure will remain unknown, thus no precipitating event provoked the seizure (1). Although epilepsy cannot be predicted in an individual, some populations are at higher risk. Ten percent of children with mental retardation (MR) and 10% of children with cerebral palsy (CP) will develop epilepsy. Fifty percent of children effected by both MR and CP will develop epilepsy (1).

Children are not the only population at risk for epilepsy. With the baby boomers expected to greatly expand the elderly population, the percentage of Americans affected by epilepsy could expand with it. Three percent of those reaching 75 years will have the diagnosis of epilepsy, with 10% having experienced at least

one seizure in their lifetime (1). Other factors such as history of stroke, Alzheimer's disease, and familial epilepsy can place an individual at greater risk for developing epilepsy. Of those diagnosed with epilepsy, 30-40% will not respond to medications and therefore be considered intractable (9-12).

Etiology/Physiology

Seizures result when neurons conduct electrical impulses in a disordered way (13) . Normal neuronal function involves the systematic firing of electrical impulses along the axons from one nerve cell to the next. Neurotransmitters are chemicals that act as messengers to transfer information across the synaptic gap to the next nerve cell. These neurotransmitters are released across the synaptic gap and taken up by the dendrite of the next nerve cell (13). When the amount of excitatory neurotransmitters outnumbers the inhibitory neurotransmitters, the cell will "fire;" thus the signal being transferred down the nervous system continues. During a seizure, these impulses do not follow the typical, systematic process. A sudden neuronal misfiring radiates through the nervous system originating in all or part of the brain (13). If enough neurons are involved in this sudden burst of neuronal misfiring, the result could be a seizure. The misguided signal transfers through the nervous system and will present in many ways, depending on the area of the brain where the misfiring occurred. If only a section of the brain is involved, the seizure could appear as modest or violent jerking, falling, confusion, staring or have other manifestations. If the entire brain is

involved in the electrical misfiring, the result could be a convulsive seizure, affecting many functions of the brain (13).

Epilepsy has a broad spectrum of etiologies that include physical damage to the brain, genetic defects, metabolic diseases and idiopathic cases in which the case is unknown. Physical damage to the brain that could lead to epilepsy includes head trauma, a cerebrovascular accident, aneurysm, or any other condition that causes an injury or necrosis to the brain. This change of structure to the brain can interrupt electrical homeostasis, thus putting the brain at risk of misfiring of the neurons (13). The field of genetics is a growing one, as the understanding of the human genome is advancing. Gene deletions and mutations that affect the excitability of neurons and contribute to epilepsy are being found which as lead to our general understanding of many idiopathic cases (14). Metabolic diseases such as urea cycle disorders can cause brain dysfunction that may result in seizures. Some of the presumed causes include energy failure, increased free radicals and possible altered neurotransmitter metabolism (15). Other cases of epilepsy are idiopathic in nature, meaning the cause is unknown. Neurons misfire and result in seizures, but the cause is unknown.

Intractable epilepsy

Epilepsy that does not respond to treatment is considered intractable epilepsy. Thirty to 45% of patients with epilepsy will fall into this intractable category (9-12).

This lack of seizure control goes beyond just a physical impairment, as social acceptance and self-esteem issues could result. Psychiatric comorbidities are common with epilepsy and may include increased anxiety, psychotic disorders, and depression (16). Individuals with epilepsy face a social stigma that can impact quality of life in many ways and this could be magnified if the person is consistently experiencing uncontrolled seizures. People with epilepsy are often viewed as violent individuals during and in between seizure events (17). The patient with epilepsy who fails to respond to medical treatment is at greater risk of personal injury, learning disabilities, social embarrassment, unemployment, inability to drive, and an overall decrease in living independently (18). In fact, the social impact for people with childhood epilepsies is more profound than with any other chronic disease state (19) . These individuals are less likely to live independently and form functional life relationships (19).

The intractability of epilepsy is poorly understood, as predictability of who *will* or *will not* respond is limited. Two subjects can present with virtually identical seizure activity and/or etiology, yet respond differently to treatment. For example, Dr. Jacqueline French reported on 2 sisters with idiopathic generalized epilepsy that developed in each during adolescence. One of the siblings was successfully treated with antiepileptic drugs (AEDs) and the other was intractable, thus failing multiple AEDs. The clinical outcomes of these siblings were very different, despite similar presentations and genetics (12). Little is known as to why some would respond and others would not. This lack of

understanding is problematic in treating persons with epilepsy, as outcomes cannot be predicted and treatment choices cannot be standardized.

Failure of the first prescribed AED increases the risk of intractability. Kwan & Brodie found that out of the 525 patients at their center in Glasgow, Scotland, that 47% of their patients became seizure free after initiation of the first AED. Only 13% became seizure free on the second monotherapy choice and a mere 1% realized seizure freedom on the 3rd choice (20). Thus, risk of intractability rapidly increases with the failure of each subsequent AED. This goes along with the common notion that after a child has failed 2 mainline AEDs, the likelihood of finding a medication to control seizures is minimal (20). Antiepileptic drugs continue to be prescribed after the failure of 3 AEDs and are often pushed beyond therapeutic levels to toxic levels in an attempt to gain some seizure control (21). This is problematic especially to the developing brain of a child. Polypharmacy is also a concern in this population, as clinicians add medications to the current one(s) to offer an attempt at seizure control (21). Not only are individuals taking multiple seizure medications at risk of adverse effects, they can sometimes realize an adverse effect of worsening seizures (22). And with some epilepsies progressive in nature, the disease state could worsen due to the intractability of the epilepsy, despite AEDs prescribed (12).

Baker, et al found that for the patient with epilepsy, the most important variable in determining quality of life is freedom of seizures (23); therefore, clinicians are

challenged to find treatment options and combination of options that control the seizures. Those with intractable epilepsy pose the biggest challenge.

History of Treatments

As expected, the historical treatment of epilepsy is as diverse as the beliefs and understandings of the disorder itself. Throughout the years, remedies have included exorcism and warding off spirits, dietary regimens, medications and surgery (11, 24-26). Though medical advancement has produced a clearer picture of the nature of the disorder, all these different treatment approaches are used today. Even the less-than-conventional ones continue to be used in some parts of the world (24).

Sacred Medicine

Antonius Guainerius in the early 15th century wrote of "sacred medicine" to treat this "sacred disease." The prescription included intake of a frog's liver, blood to smear on the patient's mouth, bile from a freshly killed dog, and urine given to him/her from the shoe of the first person to see the seizure (27). One can only imagine the fear and despair surrounding the disorder that has a treatment of such extremes. Yet, when etiology is presumed to be spiritual in nature, it seems logical to treat with spiritual tools. Still today, some religions ascribe to epilepsy as being spiritual in nature and in need of spiritual healing (6).

Anti-epileptic drugs

With emerging understanding of epilepsy came more modern treatments. Anti-epileptic drugs (AEDs) were first reported in use in 19th century.

The bromides were the first known antiepileptic drug (AED) class to treat epileptic seizures. The English physician, C.B. Radcliffe, first reported in 1860 that Sir Charles Locock had found some success with using potassium bromide for a myriad of neurological disorders, of which he included epilepsy (28). However, these drugs were not greatly utilized in treatment of epilepsy until Horase Y. Evans reported his concerns about their underutilization in an article in the American Journal of Medical Science in (28). Following this publication and a few clinical studies offering some evidence of efficacy, the bromides gained popularity and usage. Yet, these clinical studies were far from the class I studies of today and there was little drug standardization. Authors of an English review of the literature on the subject reported a minimum of 45 different preparations (28). Regardless, this served as a first attempt at treating this disease medicinally.

At the turn of the twentieth century, barbiturates were becoming more readily used in the field of neurology (29). Of this class of drugs, phenobarbital was found to have some anti-epileptic properties around 1912 (29). At this point, bromide therapy was beginning to lose its favor. In fact, around this time the

former president of the National Association of the Study of Epilepsy, W. C. Graves, described the patient on bromides as being “made to live a living death” as descriptive of their decrease in cognitive functioning while on the drug (28). Phenobarbital, too, had its sedative drawbacks, nonetheless, a second option had been added to the choices of drugs for epilepsy. This was the beginning of what could be classified as “1st generation” AEDs. These AEDs were not scrutinized by the class I, double blinded, randomized trials that the 2nd generation AEDs underwent.

A major breakthrough in drug therapies for epilepsy came with the introduction of phenytoin in the 1937 (30). In fact, this one AED revolutionalized epilepsy treatments and was the first of a flurry of new AEDs, targeting differing epilepsy types. These new medications were much needed in those days, as the 2 AEDs previously mentioned had such sedative side effects. Yet, AEDs developed in the mid 1900s had their drawbacks. For example, side effects such as gingival hyperplasia and hirsutism were seen in patients prescribed phenytoin. Digestive and liver function as well as behavioral side effects were seen with valproic acid (31, 32). Both of these AEDs are still in use today (clinical observation), despite well documented side effects.

The 1990s brought the introduction of the 2nd generation of AEDs. These drugs were subjected to more rigorous testing that was mandated by the Food and Drug Administration. Although these newer medications seemed to have fewer

side effects and appeared safer, efficacy rates have not improved (33). While pharmaceutical treatments appear easier and are more readily used in treating epilepsy than alternative therapies, they are not without disadvantages. Clinicians are challenged to effectively treat their patients with epilepsy; therefore they often will prescribe AEDs to near toxic levels in an attempt to gain seizure control (12). And the impact of AEDs on the maturing brain, as in neonates and children, is still uncertain. Animal studies have suggested that these AEDs could impact development negatively (34). While numerous AEDs are available, effectiveness of each varies by seizure type. Many AEDs are accompanied by serious side-effects such as hepatotoxicity, anorexia, weight gain, and sleep, behavioral, and cognitive disturbances (35).

Surgeries

Surgical interventions, including temporal lobectomy, hemispherectomy, corpus callosotomy, or vagus nerve stimulation, are invasive procedures and have their own disadvantages. In evaluating for candidacy of lobectomy or hemispherectomy surgeries, patients typically undergo a rigorous screening by a multidisciplinary medical team to determine if a seizure focus can be found and further removed without loss of function (36). Neuroimaging, neurophysiology, and neuropsychology disciplines contribute to evaluate a patient to determine whether a seizure focal point can be found in the brain and if it can be removed without compromising function. Some potential risks of these brain surgeries

could include decreased memory, movement, or speech functions (36). Though seizure freedom results have been reported as high as 87% for some types of resection, long-term and developmental outcomes are still unclear, especially in children (36). Corpus callosotomy surgery is a palliative treatment for intractable epilepsy, as the purpose is not to control seizures as with other surgeries, but to stop the flow of electrical discharge from generalizing (involving the entire brain) during the seizure (37). It is hypothesized that the corpus callosum is the pathway in which electrical impulses travel from one side of the brain to the other; therefore surgically disconnecting part of all of the corpus callosum has been shown to eliminate generalized seizures in up to 80% of patients in some studies (37). Yet this treatment is not without possible adverse effects, as language impairment, memory deficits, new seizure types, and other neurological effects have been reported in as many as 27% (37). Vagus nerve stimulation involves implantation of a “pacemaker” type device into the chest cavity that is connected via leads to the vagus nerve which is responsible for sending messages to the brain (38). The device can be programmed to send periodic or spontaneous electrical charges to the brain (38). One study reported 37% of patients had at least a 50% reduction in the first year of use (38). This treatment includes surgical risks of infection as well as side effects of throat pain, constriction of the throat, and voice change. In most patients these effects were tolerable (38).

Diet

Dietary treatments for epilepsy date back to the *Hippocrates* era and biblical-times when fasting was the mainstay for this disorder (13). Diets that mimic starvation have been developed and continue to be used today (13). These diets are commonly referred to as ketogenic diet, as they induce ketosis in the body. Ketogenic diets are typically high in fat and low in carbohydrate. The classic ketogenic requires a strict adherence to dietary restrictions. This diet typically requires that all foods are weighed on a gram scale to comply with a strict fat to non-fat diet ratio (13, 39). Macronutrient details must be available for each food in the diet so that meal plans can be precisely calculated to adhere to the strict diet ratio and kcalorie allotments. Such a restrictive diet can be difficult to comply with, yet for those with intractable epilepsy the tight restrictions may be merited. Ketogenic diets are used worldwide (38, 40) and researchers are fast at work to maximize this treatment's potential and uncover the mechanisms that make it effective. A more detailed overview of dietary treatments for epilepsy is covered in the next section.

The Classic Ketogenic diet

The ketogenic diet (KD) is an attractive alternative for the treatment of epilepsy. This treatment requires the patient to strictly adhere to a dietary regimen consisting of high fat, adequate protein, and few carbohydrates (13). Often this

treatment is reserved as last resort possibly due to the dietary restrictions and rigidity of the treatment (13).

History and Current Use

Historically, starvation was a mainstay in the treatment of epilepsy as described in the historical review compiled by Bailey *et.al.,* with even Hippocrates acknowledging its benefits (41). In the early 1900s, a fanatical magazine publisher of a 500,000 circulation health and physical fitness magazine brought light to the idea of fasting, believing that any disease state could be healed with enough exercise and fasting (13). Bernarr Macfadden had gained popularity and influence among his readers and even hired an osteopathic physician, Dr. Hugh Conklin, to treat patients using fasting with many disease states such as diabetes, asthma, bladder disease, prostate disease, impotence, paralysis, liver and kidney disease, eye troubles, and *epilepsy* (13). Dr. Conklin began to notice success, especially in the epilepsy patients, and begin to draw the attention of the medical community. It was not until 1921 that a practical application was introduced when R.M. Wilder first devised a high fat, low carbohydrate diet that produces ketonemia, thus mimicking starvation (42). The diet of the 1920s closely parallels the ketogenic diet used today for intractable epilepsy. It was a primary mode of treatment for epilepsy at that time, but its use waned throughout the mid-20th century with the introduction of phenytoin and newer AEDs (see Anti-epileptic Drugs above). KD has experienced a recent

resurgence of interest due to its effectiveness in children with epilepsy refractory to management with AEDs. In fact, in recent years, researchers are seeking ketogenic diet mechanisms and testing newer, less rigorous diets that produce ketonemia in an attempt to make a diet for epilepsy easier on families and more palatable (43, 44).

Composition

The ketogenic diets used today to treat epilepsy are typically high in fat, low in carbohydrate, and provide adequate protein. Ketogenic diets are typically insufficient in vitamins and minerals that need to be supplemented (13, 39). Recommendations for supplementing the ketogenic diet include a standard multivitamin with minerals (including trace minerals) and calcium with vitamin D (13, 39). Other supplements including oral citrates, selenium, magnesium, zinc, phosphorus, extra vitamin D, carnitine, MCT oil and sodium may be supplemented when indicated (13, 39). Although variations of the diet have recently been tested, the ketogenic diet of the 1920s is surprisingly similar to the classic ketogenic diet (KD) used today. The typical KD macronutrient ratio is 4:1 by weight (4g fat:1g carbohydrate + protein), which translates to a diet in which 90% of energy is derived from fat (45). Protein is often fed at the RDA level with total kilocalories reduced to 75% of requirements which permits linear growth while restricting weight gain (45). Historically, epilepsy centers have restricted fluid to 90% of needs, yet many centers have discontinued fluid restriction, as it

has not been shown to improve efficacy (13, 39). The basic composition has remained essentially unchanged over the years and is based and managed largely on convention and empirical evidence.

Efficacy of Treatment

Published findings indicate the classic ketogenic diet (KD) is more effective and has fewer side effects than currently-available pharmaceutical treatments (25, 46). Results from a recent, controlled, randomized study in the UK, indicate that the ketogenic diet is effective in children having at least 7 seizures per week (47). This study was a landmark study, as it is the first published for this treatment with a controlled, randomized design. Findings, summarized in figure 1, included 38% of children on the ketogenic diet experienced a >50% decrease in seizures, compared to the control group with 6% (47). Seven percent of the children in the ketogenic diet group had >90% improvement, compared to none in the control group. Unlike anti-epileptic drugs (AEDs), the ketogenic diet is efficacious across many seizure types (25, 46). Figures 2 and 3 summarize results from The Johns Hopkins Medical Institutes and the The Clevelend Clinic of the efficacy across seizure types. Use of the KD has also been associated with improved developmental outcomes, including significant improvement in motor skills, attention, and social problems (48), which can contribute to improved quality of life for children with epilepsy.

Vining, et al 1998 prospective study (49) at the 3 month mark, 25% had >90% decrease in seizures; 29% had 50-90% decrease; thus 54% had at least a 50% decrease (an effect). At the six month mark, 29% had a >90% decrease, 24% had a 50-90% decrease, thus 53% had an effect. At the 12 month mark, 22% had >90% decrease, 18% had a 50-90% decrease. Forty seven percent remained on the diet past 12 months. This continuation on the diet can be construed as an indicator of efficacy due to the restrictive nature of the diet, most families would not continue unless improvement was seen.

Variations of Classical Ketogenic Diet

One common complaint about ketogenic diet therapy is that compliance with the rigorous regimen is hard on children and families. In addition, some children find the classic ketogenic diet unpalatable. In recent years, new approaches have advanced to combat these issues and make diet therapy for epilepsy a little more attractive to both the patient as well as the family. These new approaches, if effective, could further increase understanding and aid in the hunt for

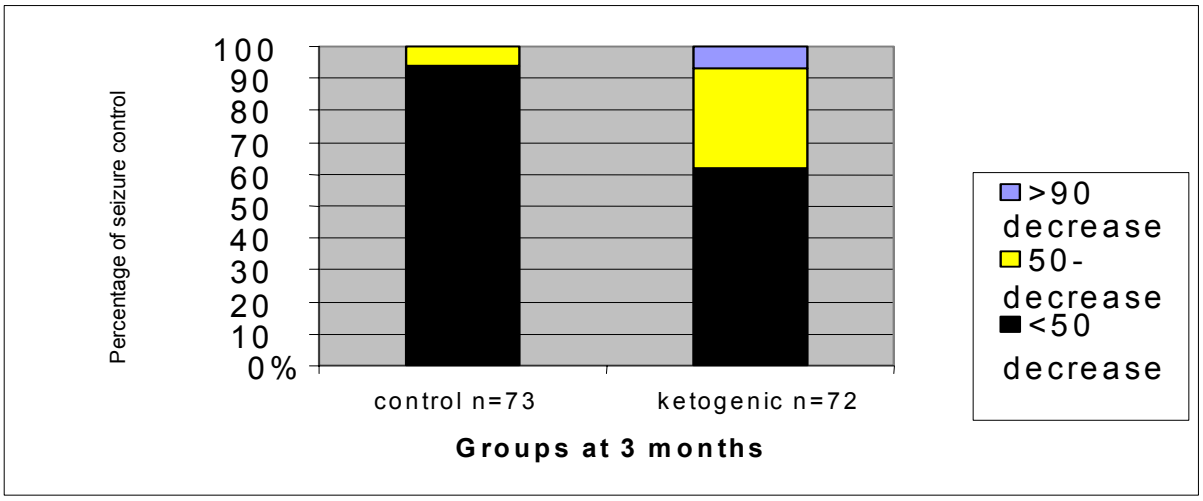

Figure 1 – Neal, et. al. (2008) (47).
A randomized trial of ketogenic diets in children at The Institute of Child Health and Great Ormond Street Hospital for Children NHS Trust. n=103.

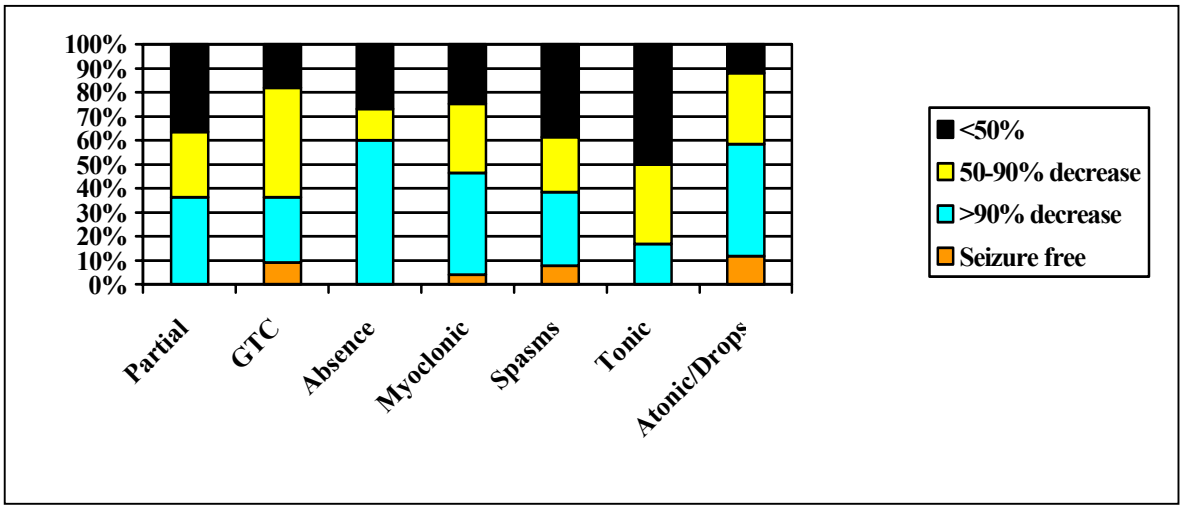

Figure 2 – Freeman, et. al. (1998)(25)
Prospective study of children with intractable epilepsy treated with the ketogenic diet at Johns Hopkins Medical Institutes, n=150.

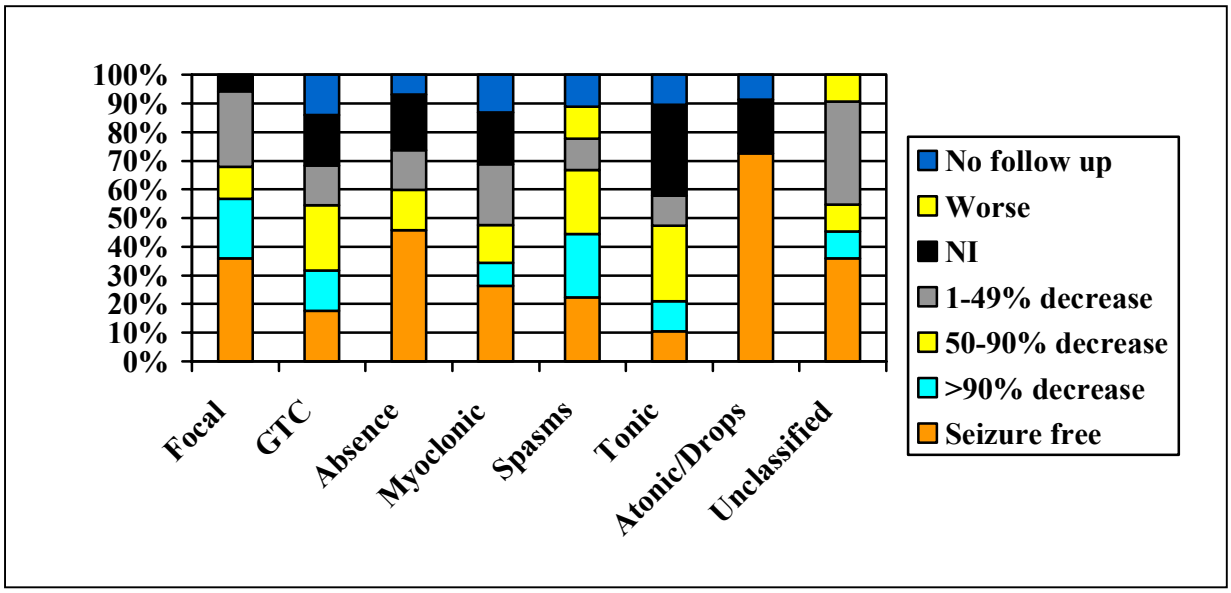

Figure 3 – Maydell, et. al. (2001)(46)
Retrospective study of children with medically intractable epilepsy treated by the ketogenic diet at The Cleveland Clinic. n=134.

Atkins

Dr. Eric Kossoff and his team at Johns Hopkins Medical Institutes have been exploring a variation of the ketogenic diet, typically referred to as a modified Atkins diet (MAD). This approach allows the patient a diet that is high in fat and low in carbohydrates, yet, unlike the classical ketogenic diet, does not limit protein or overall calories. Preliminary data suggests this diet is well tolerated and effective at controlling seizures in children as well as in adults (43, 50). The diet remains very restrictive in allowance of carbohydrate grams, yet overall is more liberal as fat and protein can be increased in the diet if desired. Foods are not pre-weighed by the family and overall administration of the diet is less regimented (43, 50).

Low Glycemic Index Treatment

Dr. Elizabeth Thiele and her team at Massachusetts General Hospital in Boston, MA, have developed the low glycemic index treatment, an alternative to the classic ketogenic diet, that liberalizes the amount of carbohydrate allowed each day, yet restricts certain carbohydrates based on the blood glucose effect these foods exert (51). In 1981, Jenkins, *et al*., first introduced evidence that different carbohydrate sources have differing effects on blood glucose when digested and absorbed (44, 52). Their preliminary findings are quite impressive, as 50% of their patients (n=20) realized a greater than 90% reduction in seizures (44). Although these and the MAD findings are preliminary and from small, uncontrolled studies, 96% of representatives from major centers from all over the world that use the ketogenic diet report offering the MAD and LGIT diets in their centers (39).

Possible Mechanisms

One of the primary focus areas of current research involves altered substrate utilization in the brain during ketosis. The brain is normally an obligate user of glucose as its primary energy source. However, glucose is in short supply during fasting or the KD and the body switches to dietary and endogenous fats as a source of energy. Fatty acids undergo beta-oxidation in the liver to yield acetyl-CoA, which, in turn, would normally enter the TCA cycle to combine with

oxaloacetate to yield ATP. However, decreased intake of carbohydrate limits availability of sufficient oxaloacetate; therefore, acetyl-CoA accumulates to a level exceeding the capacity of the TCA cycle. Acetyl-CoA molecules then condense to synthesize the ketone bodies acetoacetate (AcAc), beta-hydroxybutyrate (BHB), and acetone, resulting in ketosis. Both BHB and AcAc are exported to peripheral tissues for use as an energy source to many of them, including the brain. These ketones are transported across the blood-brain barrier via facilitated diffusion using the monocarboxylate transport system. Thus, under these conditions, the brain converts from glucose oxidation to utilization of ketones as its primary energy source (53).

Consequently, it has been proposed that ketones exert a direct anti-epileptic effect. Acetone and AcAc have been shown to suppress seizures (54, 55), while BHB has not been shown to be effective (54, 55), Elevated plasma BHB is commonly monitored as a measure of ketosis and is somewhat correlated with degree of seizure control (56), although it may simply be a marker of ketosis, indicating diet compliance. Nonetheless, it remains unknown how (and if) these ketones are involved in the diet's anti-convulsant effects. Alternatively, it has been suggested that the lack of readily available glucose is responsible for seizure suppression (54, 55). Seizure susceptibility in adult epileptic EL mice remained high when the KD was fed *ad libitum* and seizure control was only achieved when the diet was also calorically restricted to achieve a lower level of blood glucose (57). Caloric restriction has been equally effective in rats (58) and

this same phenomenon has also been observed in children (anecdotal evidence). Brain glucose uptake accelerates during seizures (59) and it is possible that the lesser availability of glucose translates to a lack of readily available energy to support neuronal membrane excitability.

It is also possible that the KD alters neurotransmitter synthesis and action. The brain utilizes ketones to regenerate acetyl-CoA, which then enters the TCA cycle to produce ATP (53). When carbohydrate availability is limited, oxaloacetate is depleted and, thus, no longer available for transamination of glutamate to aspartate, an excitatory neurotransmitter. As a consequence, more glutamate becomes available for synthesis of GABA, the primary inhibitory neurotransmitter (60). Indeed, modulation of GABA is one mechanism by which AEDs exert their effects (35). Therefore, it is conceivable that efficacy of the KD is elicited, in part, via neurotransmitter modulation.

Recent research indicates that metabolic changes occur in the brain as a result of the KD. Bough, *et. al.,* found the seizure threshold lowering effect of the KD can take weeks to develop in rats (61), thus mirroring the common clinical trial of at least one month to determine response (anecdotal observation). Since the effect appears somewhat delayed, developmental changes induced by gene expression changes could be involved. Bough and his team further used microarray technology to study hippocampal gene expression changes induced by a KD in non-epileptic rats. Their findings included a significant up-regulation

of metabolic genes in the hippocampus after KD treatment (61). Similarly, Noh *et.al.,* found similar effects of the KD on gene expression in the hippocampus of non-epileptic rats using pooled samples (62).

Alterations in gene expression due to epileptic activity have also been studied (63) as well as AEDs (64). However, the effects of the KD on gene expression in a natural model of epilepsy have not yet been determined. Measuring KD-induced differentiation of specific metabolic genes in an epileptic model could begin to bridge the gap to clinical by confirming effect in an epileptic brain. Possible treatment options could be perfected or developed when more is understood about the metabolic processes occurring in the epileptic brain when treated with a KD.

Animal Models of Intractable Epilepsy

Mouse Models

Finding a suitable mouse model for studying epilepsy can be challenging, as most models used are not *epileptic* mice and have seizures induced by various techniques or chemicals. One commonly used technique to test seizure threshold is the 6 Hz test. This technique involves placing electrodes on the cornea of the mouse and administering electrical shocks to measure seizure susceptibility (65, 66). Current intensity values are chosen utilizing a stair-stepping method in which intensity is set dependent on how the previously tested

mice responded. Mice are euthanized following testing. Although the ketogenic diet has been evaluated and shown effective using this model (66), the seizures are not naturally occurring as in the human disorder of epilepsy.

Another commonly used technique to study seizure disorders is chemical induction of seizures. Researchers have used various chemicals such as bicuculline, picrotoxin, kainate and gamma-butyrolactone to induce seizures (67). These chemicals are typically administered subcutaneously in varying doses, depending on the level needed to induce seizures. Each of these chemicals above have been used in rats in exploring the ketogenic diet (67), yet again, natural seizure thresholds cannot be evaluated as the seizures are induced and not naturally occurring in the animal model.

EL Mouse

The EL/Suz (EL) mouse model is particularly suited for studying diet manipulation for epilepsy due to the naturally occurring seizure activity observed in this model without the use of chemical agents or extreme stimuli (such as tossing or rapid cage shaking). This model was first described in 1959 when Imaizumi, et.al., observed convulsive seizures in a hydrocephalic mice strain after the mice endured a sudden stimuli such as tossing. Imaizumi's group went on to inbreed the seizure susceptible mice to develop a mutant model of generalized epilepsy (68). This mouse model develops seizures in combination

with aging, repetitive stimuli such as handling during cage changes, and previous seizures (69).

The use of the EL mouse is well defined in the literature and has been previously shown to respond well to treatment with the KD (57). Male EL mice have been reported to have instances of sudden death (70) and female EL mice have been reported to show poor maternal care of offspring (71). Therefore, growing and maintaining a colony of this model can be challenging, yet cross-breeding techniques can be utilized to increase colony size.

Applicability to humans

The EL mouse is particularly well suited for studying epilepsy as it represents common presentation of this disease state, secondarily generalized epilepsy (69). Seizure susceptibility is inherited as an autosomal dominant trait in these mice and develop in response to environmental stimuli (69).

With the advancement of genetic science has come understanding of some of the genetically linked epilepsies. Researchers have found that gene defects of some of epilepsy syndromes are simple, single gene defects (72). But these monogenetic epilepsies are rare. Most human epilepsies are thought to be multifactorial with more than one gene involved and/or multiple contributing environmental factors (73). The combination of genetic and seizure susceptible environmental factors are hard to duplicate in an animal model. The EL mouse

combines both of these elements and therefore is well suited as an applicable model for the majority of generalized epilepsies. Electrical shock and chemical induced seizures do not progress from physiological processes. Therefore, the EL mouse model is a prime model for investigating the ketogenic diet, as its naturally occurring epilepsy better mimics human epilepsies.

Dietary Design in Ketogenic Diet Research

Experimental Diets

There is little question that the KD works, but maximizing its clinical utility requires an evidence-based understanding of *how* it works. An essential part of testing this treatment in the animal laboratory is designing experiments that are highly controlled so that findings can be accurately interpreted. Previously, researchers have used ketogenic diets provided by Zeigler Bros., Inc. (Gardners, PA) and from Bio-Serv, F3666 diet (Bio-Serv, Frenchtown, NJ)(66, 74, 75). The Zeigler ketogenic diet was used by Mantis, *et al.,* in a previous study to evaluate efficacy of ketogenic and calorie-restricted diets, using the EL mouse model. The diet used in this study consisted of 100g fat, 0g carbohydrate, 128g protein, and 109g fiber per kilogram along with a standard diet for the control group (57). When feeding the calorie-restricted diets, Mantis, *et. al.,* simply gave *less* of the standard and high fat diet to the mice. Therefore, not only did the mice receive less kcalories, they received less protein, fiber, vitamins, minerals and other ingredients. Therefore, when seizures were decreased in the calorie-

restricted groups, they concluded that kcalorie restriction induced this reduction in seizures (57). Yet, with so many nutrients decreased in the diets the mice received, it is unclear exactly which nutrient deficiency could have a causative role in the seizure reduction. Thus, this lack of tight control in study diet design could have led to unclear findings.

Similar methods were reported in studies using the F3666 diet (Bio-Serv, Frenchtown, NJ)(66, 74, 75). The diet is commonly reported to a 6.3:1 fat to non-fat ratio, yet the data sheet provided by Bio-Serv calculates to be 8.66:1 fat to non-fat ratio. In studies reviewed utilizing this diet formula, again, calorie-reduction is achieved by simply reducing the amount of chow given; therefore reducing the previously mentioned nutrient. Further, extreme diet fat to non-fat ratios are not applicable to humans. A diet of over 4.5:1 is rarely given to children and when it is prescribed, it is usually only limited to a few months due to possible adverse effects (39).

Karin Borges and her team at Texas Tech University Health Sciences Center came a little closer to designing a controlled diet in her most recent study, *Anticonvulsant profile of a balanced ketogenic diet in acute mouse seizure models,* Samala*, et al*., (2008) (76). Diets in this study included ketogenic diets at a 4:1 (TD.06233) and 6:1 (TD.07797) ratio. Borges took a novel approach in diet design by matching vitamins, minerals, and antioxidants across all groups relative to their caloric densities. Yet, with the controls used here, protein and

fiber were *not* matched among groups. Interestingly, her results were diverse from other findings using similar methods (76). Four different mouse model techniques were used (6-Hz, and 3 different chemicals), and ketogenic diet efficacy was only seen in the 6-Hz model at a 6:1 fat to non-fat ratio, not in the 4:1 ratio (76). Although the researchers in this study did not draw conclusions in this regard, it is interesting to note that previous studies *without* standardized amounts of vitamins, minerals, and antioxidants among groups had different findings from this novel approach in diet design.

As scientists are fast at work to uncover the mechanisms that bring about the success of the ketogenic diet, examining experimental design is the key to insuring results can be interpreted accurately. When looking for mechanisms facilitated by diet, diet design is of great importance. In previously mentioned study designs, several nutrients are not consistent across groups. One that has potential importance is the macronutrient, protein. In studying the ketogenic diet, much focus is given to *adding* fat and *limiting* carbohydrate. Little attention is given to maintaining adequate protein. Yet, in the clinical administration of the diet, much attention is given to this vital nutrient for tissue maintenance and growth (in children). In fact, younger children and infants are sometimes restricted to a lower ketogenic diet ratio of 3:1 due to higher protein needs per kilogram (39). Thus, clinicians determine the need to provide adequate protein more important than maintaining the gold standard ratio of 4:1 (4g fat to 1g non-fat). Further, protein can effect overall well being, including brain function. In

fact, Feria-Velasco, *et. al*., recently reported that low tryptophan and protein levels during development can result in increase seizure susceptibility in adult rats (77). Low protein intakes have also been linked to glutamatergic activity in the brain of young albino rats resulting in damage to the brain (78).

In low carbohydrate ketogenic diets, including fiber can be challenging. Fiber is an important nutrient in the human diet and high intake has been linked to increased health benefits (79). Dietary fiber can also affect nutrient absorption, such fat (80). Fiber can also adversely affect glucose levels, decreasing carbohydrate absorption rates (81). In studying ketogenic diets, tight control of any element that could affect blood glucose is critical. In manipulating diets and evaluating changes brought by this manipulation, dietary fiber needs to remain the same to aid in accurately interpreting results. Vitamins and minerals play an important role in maintaining human and animal health. Lack of nutrients such as B-vitamins have been shown to adversely affect learning in growing brains (82). Vitamins and minerals play an active role in many metabolic pathways (83). Limiting them could hinder pathways, or alter efficacy of the pathways. These nutrients need to be maintained at constant amounts when designing experimental diets as to eliminate confounding factors. Thus, for experiments to have a clinical application, experimental designs need to be applicable to humans and utilize tight controls so that results can be properly interpreted and applied.

CHAPTER III
MATERIALS AND METHODS

Animals

Procurement

This animal study was fully conducted in accordance with the NIH *Guide for the Care and Use of Laboratory Animals*. The Institutional Animal Care and Use Committee (IACUC) at the University of Tennessee, Knoxville, TN, approved protocols for both breeding animals for this study as well as the protocol used to examine the effect ketogenic and calorie restricted diets in epileptic mice. All investigators, laboratory personnel, and volunteers in contact with these animals were provided training, passed the examination for "Working With the UT Knoxville IACUC" course (ResearchTraining.org), and either provided a waiver or enrolled into the University of Tennessee's occupational health program.

Six breeding pairs of inbred EL/Suz (EL) were donated by Dr. Thomas Seyfried from his animal laboratory in the Department of Biology at Boston College (Boston, MA). They were approximately 6 weeks old when they arrived at the laboratory located at 1215 W. Cumberland Avenue, Knoxville, TN, Room 319. These EL mice were born on 5-21-06 and 5-22-06. The mice traveled in a cardboard animal box containing a wet gel used to keep them cool. The animals were separated by sex and clearly earmarked for identification. Cage cards from BC accompanied the mice, providing information needed for separating the mice

into sibling x sibling breeding pairs. Animals and animal chows were handled utilizing sanitary procedures including clean work areas and fully gloved hands.

Eight female and 4 male inbred C57BL/6J mice (B6) approximately 6 weeks old were obtained from Jackson Laboratory (Bar Harbor, ME). These mice arrived in a container similar to the El/Suz mice, separated by sex. Upon arrival, these mice were group housed as described above by sex until ready for breeding.

Housing/general care

Upon arrival, all mice were group housed in plastic shoebox cages with wire bar lids and lined with pelleted paper or beta chip bedding (both, Harlan-Teklad, Indianapolis, IN) to absorb urine and to provide a means for the animals to burrow. The mice were given *ad libitum* access to water and AIN-93G purified pelleted chow (Dyets, Inc., Bethlehem, PA). The animals were separated by sex and breed and a maximum of 5 adult mice were housed per cage. All cages were clearly marked with an index card listing the mouse strain, the IACUC protocol number, the principal investigator's (PI) name, PI's University of Tennessee telephone number, PI's home telephone number, graduate student's name, graduate student's mobile telephone number, source of the mice, date of birth of the mice, and mouse number (when applicable). Cages were placed on a portable stainless steel rack of shelves maintained in a 3-foot by 6-foot negative pressure cubicle within the animal laboratory facility, which was labeled

Cubicle 8. The animal laboratory was equipped with a clean/dirty airflow system to decrease likelihood of cross contamination between animals housed within the facility. Temperature was maintained at 24 degrees Celsius and relative humidity was maintained between 45-75% by an onsite dehumidifier. The facility was maintained on a timer controlled alternating 12:12 hour light-dark cycle. The interruptions of this light-dark cycle was necessary for monitoring during the course of EL breeding, when the mice were checked periodically throughout the dark, 12 hour night cycle. During these times, the cubicle light was turned on for less than 15 minutes for checking and tending to any newborn pups. All mice were allowed *ad libitum* access to food prior to study randomization. All EL and B6 breeders were fed AIN-93G purified pelleted chow (Dyets, Inc., Bethlehem, PA) prior to randomization. Chow pellets were placed on the floor of the plastic shoebox cages of nursing pups beginning at approximately 14 days old until they were weaned. All mice, prior and after randomization, were allowed *ad libitum* access to water provided by a water bottle with sipper tubes placed on each cage lid. Cages were changed weekly on Wednesday mornings at the end of seizure testing, unless contraindicated due to breeding and birth of pups.

Due to the stimulation induced seizure susceptibility of the EL mouse strain, minimal disturbance was merited. Therefore, only the PI's laboratory assistants and this graduate student were allowed to handle the mice or disturb the cages in Cubicle 8. The only exception included the changing of water bottles by the animal laboratory staff if the bottles were less than ½ full. These bottles were

checked daily. A sign was placed on the door of Cubicle 8 that reminded walkers-by that the mice were stress sensitive animals and should not be disturbed.

Female HSD:ICR mice were maintained in Cubicle 8 as sentinel mice for periodic testing. During cage changing, a pinch of dirty bedding from each of the mouse cages in Cubicle 8 was placed into a clean cage. The sentinel mice were then transferred to this cage to purposefully spread any microbial contaminant that may be found in our mice. These sentinel mice underwent periodic serology tests, scotch tape tests and fecal exams to test for contamination. The results of the sentinel mice testing were unremarkable in Cubicle 8 during the time period of the experiments describe herein.

Breeding

The goal in developing the EL/Suz (EL) colony was to breed and grow 20 female EL mice that would prove seizure susceptible (see seizure testing below) to qualify for the current study. As described in the above literature review, EL colonies can be challenging to grow due to the poor maternal care for pups by the EL dams. Cross-fostering techniques were utilized to optimize success of breeding 20 female EL mice to supply the animals needed for the study. Black-6 (B) female dams have previously in this animal facility proven suitable to utilize in cross-fostering the EL pups, and thus, were employed here.

To initiate breeding, female B6 mice were placed in clean cages, 2 per cage, and then one B6 sibling or cousin male was placed in the occupied females' cage for group breeding. Males remained in the cage until female mice displayed abdominal distension at which point the female mice were then housed singly. Pregnant female mice were provided nestlets (Fisher Scientific, Atlanta, GA) made of non-ingestible pulped virgin cotton fiber to aid in nest preparation. B6 litters were typically delivered approximately 21 days after the males were placed into the females' cage. Upon delivery, mice were left undisturbed (no cage changing) for at least 3 days, unless fostering procedures were merited. The timing of breeding the B6 mice was determined based on expected pairing of EL mice. At least one B6 female began the breeding cycle 3 days prior to an EL mouse pairing in an attempt to have delivery of the B6 pups 1-3 days prior to deliver of EL pups. Our preference was to breed two B6 females prior to each EL pairing, as to maximize the potential of having an available foster dam with each EL litter.

Female EL mice were single housed in a clean cage prior to breeding. A sibling male EL mouse was placed into the female cage for breeding. Mice were allowed *ad libitum* access to purified chow and water during the breeding cycle. When female EL mice displayed abdominal distension, the male EL mouse was removed and the female was given a nestlet for building her nest. A green sticker dot was placed on the cage card to indicate that a pregnant mouse

resided there. Laboratory assistants began checking on the mice every 4-6 hours, throughout the day and night, on day 19 of breeding and every day thereafter until the litter had been dropped.

As expected, the EL dams did not exhibit strong maternal skills. Typically newborn EL pups were found scattered about the cage and cold to the touch. If rescue of these pups was not timely, the pups were already deceased and/or partially consumed by the dam.

Cross Fostering

Upon discovery of a surviving EL litter, pups were cross-fostered to a nursing B6 dam. Dr. Gary Truett, University of Tennessee Assistant Professor in the Department of Nutrition, provided training on euthanasia of neonate mice by decapitation. Neonate mice do not respond well to CO_2 inhalation (84) as is standard in euthanasia in older pups and adult mice, therefore quick decapitation facilitates a quick and humane means of euthanasia to neonates. When a surviving EL litter was found, a B6 nursing dam was selected to become a foster dam to the EL litter. These B6 litters were typically 1-5 days old when cross-fostering occurred. The B6 litter was completely removed from the B6 dam's nest, while leaving the nest intact. During this time, her shoebox cage was returned to Cubicle 8 with the doors closed to reduce her awareness of euthanasia of the pups. The pups were taken away from Cubicle 8 and quickly decapitated with surgical scissors. The carcasses were disposed of in the

carcass freezer in the animal laboratory from which they would later be taken for incineration. After the changing of gloves and removal of any blood products in the area, the EL litter to be cross-fostered was gathered into gloved hands. The pups were held for approximately 2 minutes or until they were warmed to the point of continuous movement (cold pups have very little movement). Pups that did not move after several minutes of warming were considered expired and their carcasses were removed from the litter and disposed of. When the new EL litter was warmed, it was placed gently into the B6 foster dam's nest. Often the B6 foster dam would scatter the new foster EL litter across the cage and then re-gather them back into her nest. The B6 dams used in this breeding experience proved to be good foster dams if fostering occurred anywhere between 1 and 5 days after delivery of her own pups. Thus, acceptance of the EL litters by the B6 foster dam was typically good.

Weaning/Ear Marking

EL mice were nursed and remained with the B6 foster dam until they were weaned at 4-5 weeks of age. At weaning, litter mates were housed by sex and ear marked for identification. At this early age, differentiating between the genders can be difficult. Therefore, occasionally EL litters remained together for an additional 1-2 weeks until the genders could be determined at which time they were separated. The ear marking technique used was ear punching, which places one or more small notches or holes in the ears at various locations as

defined previously by Dickie 1975; Ingalis 1980; Stark and Ostrow 1991. The ear-punching instrument (BTY Co., Bay City, MI) used was washed with soapy water and then sanitized with quaternary ammonia before and between uses on each mouse. This procedure requires no anesthesia, as nerve endings are few in the ear lobes (pain is therefore minimal). Offspring of EL breeders were numbered beginning with one (1) and numbered to fifty-five (55), without regard to litter affiliation or sex.

Experimental Design

The EL/Suz (EL) mouse model is a well-established epileptic mouse model. These animals were descendants of mice received from J. Suzuki at Tokyo (69).

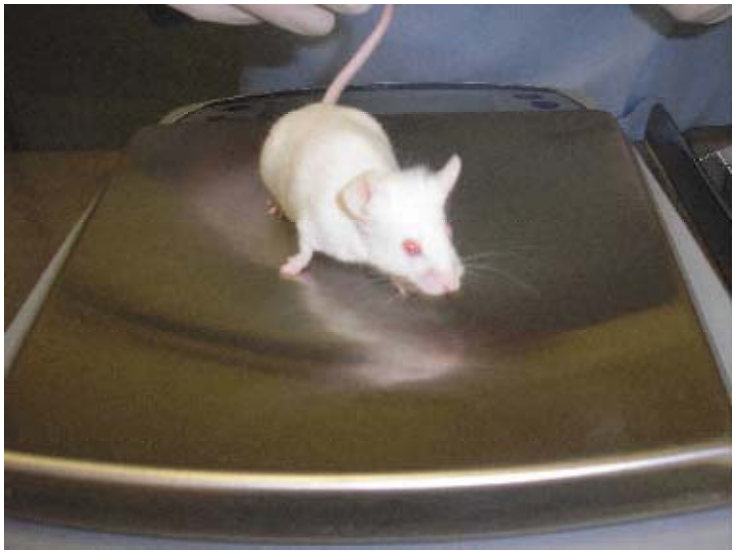

Figure 4 – EL/Suz mouse bred at the University of Tennessee, Department of Nutrition Picture courtesy of Katherine Teague.

Due to poor nursing and pup retrieval previously observed in females of the EL strain, the offspring of these breeding pairs were cross fostered shortly after birth to nursing C57BL/6J mice (B6) dams who along with their same B6 male mates were purchased from Jackson Laboratories (Bar Harbor, ME). These B6 foster dams continued to nurse and care for these EL pups until they were weaned between 4-5 weeks of age. Upon weaning, the EL pups were group housed by sex in plastic shoebox cages with wire rack lids and standard bedding and kept on a 12-hour light/dark cycle. The mice were fed a semi-purified AIN93G pelleted rodent chow and were provided water *ad libitum*. Dietary intake was measured and recorded for the duration of the study. Weekly seizure testing began at weaning (+/- 3 days) and the mice were kept to minimal disturbance (no handling) with the exception of weekly seizure testing. Shortly after the seizure testing, the mice were weighed and placed into clean cages. This was the only disturbance they experienced other than passers-by and occasional water bottle changes.

This weekly seizure testing, as described under the *Seizure Testing* section below, occurred on Wednesdays between 7:30am-11:00am. Body weights were obtained each week after the seizure testing was complete and the mice were placed into clean cages. At 15-29 weeks of age when seizure consistency had been confirmed by a consecutive 2 week seizure score of 4 or 5 according to established scoring techniques, female mice were weighed, randomized into one of four diet treatment groups (control, control-calorie-restricted, ketogenic,

ketogenic-calorie-restricted), and housed in individual cages. Twenty female mice were used in the study and were placed into randomized groups based on the date they met study criteria and mouse ear mark number given at weaning. Most male mice were euthanized or maintained for breeding. Male mice were also randomized (n=3) for potential inclusion in the event that the colony was unable to produce the 20 females required. The animals were housed at the University of Tennessee Nutrition Department animal facility located on the third floor of the Jessie Harris Building on the main campus.

Qualifications for Study and Mice Used

Female EL/Suz (EL) mice that were at least 100 days old and were seizure susceptible during the 2 previous seizure testing sessions were included in this study. Mice were considered seizure susceptible if they had a level 4 or 5 seizure during a seizure testing session (see seizure testing). Male EL mice were excluded from this study due to reports of premature death caused by urinary retention. Mice under 100 days old were excluded due to rapid growth during that time in development. Mice that did not have a level 4 or 5 seizure (see *seizure testing*) during the prior seizure testing session or in the session before one week prior to that one were excluded.

Randomization Method

Due to the challenges of breeding an El colony (see *Breeding* above), not all mice in the study were ready to be randomized at the same time; therefore they were staggered in placing them in the study. Once mice were at least 100 days old and had experienced seizures for 2 consecutive weeks during seizure testing, they were randomized and placed into the study. When multiple mice became ready for the study, their identification numbers were numerically lined up. Treatment groups were lined up in this order: Control (C), control calorie restricted (CCR), ketogenic diet (KD), and ketogenic diet calorie-restrict (KDCR). At the first randomization, 7 mice were placed into the study. Mice to be randomized were lined up by number and 2 at a time (in numeric order) were placed in groups. The group selection began with control and proceeded with control-calorie restricted, ketogenic, and then ketogenic-calorie restricted. Thus, EL2 and EL3 were place in C, EL7 and EL9 were placed in CR, EL10 and EL14 were place in KD and EL16 was place in KDCR. Subsequent groups that were randomized at later dates were placed in a like manner.

Seizure testing

Dr. Thomas Seyfried, Professor in the Department of Biology at Boston College (BC) provided a short video clip of an EL/Suz (EL) mouse experiencing a seizure along with a description for each grade in their established grading scale. During a visit to Boston College, this graduate student and the PI were

welcomed into Dr. Seyfried's laboratory and were trained to conduct seizure testing on EL mice. With permission, a video recorder was used to tape this training session for future reference as well as for the purpose of sharing the session with the collaborating pediatric neurologist, Dr. Christopher A. Miller of Child Neurology Services in Knoxville, TN.

Seizure testing and susceptibility scoring was conducted as previously established in this model by Todorova *et. al.,* and summarized in figure 6 (85). Seizures were precipitated by repetitive handling and simulation of stress associated with cage changing. The testing session was made of 2 trials, separated by a 30 minute resting period. Each trial consisted of 2 testing phases. During the first phase, the mouse was held by the tail for 30 seconds (s) 10-15 cm above the bedding in its home cage. The mouse was then placed in a clean cage for 2 min. The mouse was held again (second phase) for 15 s prior to being returned to the home cage. This concluded the first trial. A second trial was conducted after 30 min had elapsed. Mice developing an epileptic seizure during holding were placed immediately in one cage or the other, depending on the phase of testing. Mice experiencing a level 4-5 seizure during the 30 s testing phase as defined by Todorova *et. al.,* (85) was not retested for the 15 s phase. Regardless of seizure activity during the 1st trial, the mice were undisturbed during the 30 min resting period and then retested for the second trial. Mice were tested weekly during the duration of the study. Seizure susceptibility scoring as established by Todorova *et. al.* (85) was used by

observing mouse response during the testing procedures. Mice were considered seizure susceptible if they experience a generalized seizure during seizure testing (score of 4 or 5). Generalized seizures in the mice involve loss of postural equilibrium and consciousness, together with excessive salivation, head, limb, and chewing/swallowing automatisms. Mice limited to vocalization and twitching without progression to generalized seizure were not considered seizure susceptible for the purpose of this study. Thus, only seizures affecting the entire brain were considered in our study, eliminating partial seizures. All testing activity was video recorded for verification. A summary of severity numbers used in the scoring is below. Dr. Christopher A. Miller, MD reviewed the seizure protocol established by BC on the video clip from Boston College as well as the video footage taken on the visit to Boston College. Dr. Miller further visited our

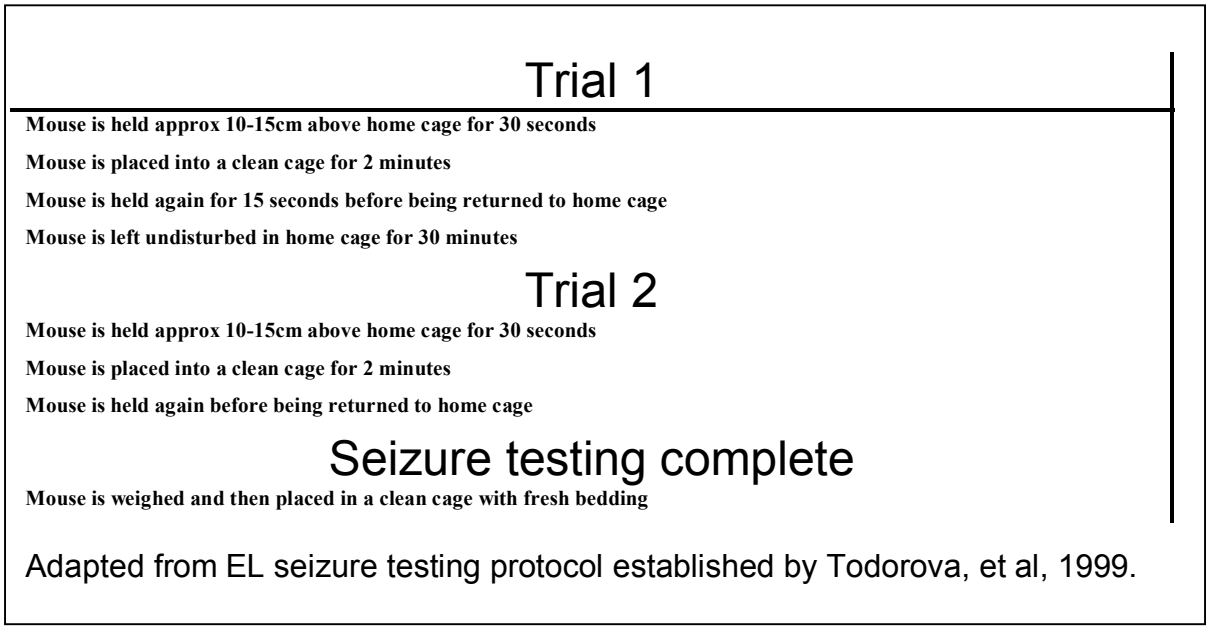

Figure 5 – EL seizure testing protocol established by Todorova, et. al. (1999)(85).

animal laboratory to verify that the grading of seizures was consistent with the grading system established by Todorova *et.al* (85).

At weaning (4-5 weeks old), EL pups began weekly seizure testing on Wednesday mornings between the hours of 0730 and 1100 in the common area of the University of Tennessee, Department of Nutrition animal laboratory. Food was removed 2 hours prior to seizure testing. Food in the cages of the mice that were eating *ad libitum* was gathered from the wire bar lid and placed in an aluminum bowl that was marked with the number of the mouse. The aluminum bowl was then placed back down on the wire bar lid. Thus, the bowl provided a barrier for the mice and the food was not accessible to the mice. The petri dish was removed from the cages of mice eating the high fat paste chow. The portable stainless steel rack of shelves on which the mouse cages set was rolled from Cubicle 8 to the common animal laboratory workspace. All counter tops used during the seizure testing and other activities were cleaned with quaternary ammonia. Group housed mice were separated by groups of two into cages. Earmarks were read and the cages labeled so that mice were identified and seizure data could be maintained for individual mice. To facilitate reading of an ear mark, the mouse was gently lifted by the tail and placed on a fisted hand, with freedom to walk around the fisted hand (mouse was not constrained other than holding of the tail). While the mouse explored the fisted hand, the laboratory assistant could easily view the ear mark and identify the mouse. Each mouse was handled in the same way even if the housing arrangements where

such that the mice did not need to be separated, thus each mouse received the same low level stimulation consistently before the seizure testing.

The seizure testing station was a table in the middle of the common area in the animal laboratory facility. The laboratory assistants stood beside the table and a stainless steel portable shelf was placed behind them as a temporary shelf to hold the wire bar lids of the cages of the mice being tested. All mice were seizure tested at the same location in the animal laboratory each week. The 2 laboratory assistants worked side-by-side, each responsible for testing a maximum of 2 mice at a time (total of 4 mice were typically tested at a time). An additional laboratory assistant was responsible for recording seizure data in a notebook and monitoring the timer during the seizure testing. The timer used was a Fisherbrand traceable count down timer (Fisher Scientific, Atlanta, GA) with triple line LCD with accuracy to 0.01%. This graduate student was in charge of grading the seizures of the mice and recording video of the session. This graduate student was uniquely qualified to grade the seizures in the mice due to the above-mentioned training at Boston College as well as the verification of grading obtained from Dr. Christopher Miller.

Seizure severity was measured on the scale of 1 to 5, with 1-3 signifying characteristics of a partial seizure and 4-5 signifying characteristics of a generalized seizure. See table 1 for a summary of the seizure grading. A grade 1 seizure was characterized by squeaking; grade 2 included immobility, blinking

and mild facial clonic (rhythmic) activity. A grade 3 was given if the mouse displayed a catatonic tail. A grade 4 seizure was characterized by forelimb rhythmic movements. A grade 5 was given to any mouse that experienced a generalized tonic convulsion involving a lost of posture and full body jerking.

For this study, a mouse considered having a generalized seizure was considered to be seizure susceptible. Further, a mouse scoring less than 4 would be considered non-susceptible to seizures for that testing session. Mice achieving a generalized seizure (grad 4 or 5) typically began with some of the lower scoring characteristics such as squeaking, immobility, twitching and catatonic erect tail and then progressed to severe forelimb clonus and generalized convulsion as evidenced by loss of postural equilibrium, excessive salivation and severe jerking.

Mice experiencing a level 4 or 5 grade seizure during the 30 second phase of the seizure testing were not re-tested in 15 second phase of that same trial. They

Table 1 – Seizure Severity Scores

Seizure Severity Scores

Score	Characteristics
1	**Squeaking**
2	**Immobility, blinking, mild facial clonus**
3	**Catatonic posture with erect tail**
4	**Forelimb clonus**
5	**Generalized tonic convulsion**

Adapted from EL seizure testing protocol established by Todorova, et al, 1999

were, however, retested in Trial 2, regardless of whether or not the mouse had a seizure in the first trial. This graduate student determined whether or not to retest a seizure during the same trial if the mouse had a level 3 seizure. If the mouse appeared still and slow to respond after the 2-minute resting time was complete, it was not retested in the 15-second phase. If the mouse recovered quickly from the seizure and was exploring the cage, the mouse would be retested during the 15-second phase.

After the seizure testing was complete, the mice were individually weighed on a Denver Instrument P-6001 gram scale with precision to 0.1 gram. An empty shoebox cage was placed onto the scale and the scale was tared. The mouse was then placed into the shoebox and the weight was recorded into a notebook and maintained on a MS-Excel spreadsheet. The mouse was then placed into their clean cage and returned to the stainless steel shelf.

Before randomization, mouse intake was measured by weighing chow amounts each Wednesday morning after the seizure testing was complete. An aluminum cup was place on a Mettler Toledo PG802-S scale with a precision to 0.01 gram and the scale was tared to zero. The chow for each cage had been gathered from the wire bar lid 2 hours before seizure testing began and placed in an aluminum cup that was temporarily stored on top of each cage. Each cup of food was taken to the Mettler scale and weighed by pouring the chow in the tared aluminum cup. This amount was recorded each week into a notebook and

maintained on a MS-Excel spreadsheet. Mice that were randomized into the control group were allowed to eat *ad libitum*; therefore their chow was measured in this same manner.

Blood Collection

All blood collection occurred via submandibular vein after seizure testing on Wednesday mornings, with the exception of the final collection with occurred at sacrifice on Saturday mornings. This collection occurred between 1 and 2 hours after the seizure testing was complete, to assure blood glucose levels had normalized in the event that the mice had experienced seizure activity during the testing. The final blood collection occurred just seconds before decapitation which occurred on Saturday mornings. Therefore, no seizure testing had occurred the day that the final blood was taken.

Blood was collected by utilizing the submandibular vein bleeding method. The submandibular vein drains from the brain as well as the cheek pouch, thus giving a rich supply of blood. This vessel is accessed by piercing the vein with a sharp object such as a lancet, needle or scalpel. Pain is minimized with this procedure. This graduate student was trained to use the technique by Dr. William Hill, Clinical Assistant Professor from the University of Tennessee Office of Lab Animal Care. Golden Rod animal lancets (Medipoint International, Inc., Mineola, NY) were used to access this blood vessel. This lancet was specifically designed for this technique of blood collection. During the collection, mice were held

securely and the lancet was used to make a quick, perpendicular puncture to the submandibular vein. The blood was collected into a BD Microtainer plasma separator tube (Fisher Scientific, Inc., Atlanta, GA) and quickly placed on ice. Pressure was then applied to the cheek of the mouse with a Fisherbrand 2x2 sterile woven gauze pad (Fisher Scientific, Inc., Atlanta, GA). Quantity of blood collected was limited to 200 µl, consistent with the University of Tennessee IACUC recommendations limiting the amount of blood taken from an animal to the equivalent of 1% of body weight. As a visual guide for collecting blood, a sample tube was prepared with 200µl of water as an upper limit quantity of blood to collect at one setting. This quantity was selected as all mice in the study exceeded 20g, thus 200µl served as a safe, conservative blood quantity with slight room for error.

Extracting Plasma

Whole blood was centrifuged in a Sorvall Biofuge Fresco micro-centrifuge (Thermofisher Scientific, Asheville, NC) at 6,000 x g for 10 minutes to obtain plasma. The plasma was collected by a Rainin pipet and placed in micro-centrifuge tubes (Dot Scientific, Flint, MI) and was quickly placed on ice. The tubes were marked with the date and mouse number and the aliquots were stored at – 80 degrees Celsius until time for analysis. The plasma samples were stored less than six months from the time they entered the freezer until they were used for running assays on the samples.

Plasma Assays

Glucose

Plasma samples were thawed from the deep freeze to room temperature. The glucose reagent was prepared and 50ml of sterile deoionized water was loaded into a cylinder to make up the solution. Cuvets were set up for standards and samples to include duplicates of each. Nine hundred µl of glucose reagent was added to each cuvet, including standards. Standards were prepared with the following glucose concentrations: 0 mg/dl (water blank), 25 mg/dl, 50 mg/dl, 100 mg/dl, and 200 mg/dl. Six µl of standard was added to the standard cuvets. Samples were diluted using 4 µl of deionized water and 2 µl of plasma sample to each cuvet (including duplicates). All samples were mixed using a vortex for several seconds. The samples and standards were allowed to incubate at room temperature for 10 to 15 minutes. Samples and standards were then analyzed using a Genesys 5 Spectrophotometer. Data produced by the spectrophotometer was then converted using a MS-excel spreadsheet. The 2 blank standards were recorded and then the standard dilutions and sample readings were recorded. Then the readings produced by the spectrophotometer for the blanks were subtracted from the readings from the standards to give an adjusted reading. This same adjustment was made for the samples (for both blanks). Using the adjusted standards numbers, a slope, a y-intercept and the square of the Pearson product correlation coefficient were calculated. For each sample, the two converted numbers (sample minus blank) were averaged together and that average was used to calculate a raw glucose score. The

formula used for this calculation is as follows: (average sample number minus y-intercept)/slope. This raw glucose score was then multiplied by a dilution factor of 3 to yield the final glucose number that is read as mg/dl.

Beta-Hydroxybutyrate

Beta-hydroxybutyrate (BHB) was measured as a marker of ketosis. BHB was measured using the Stanbio StatSite® Analyzer (Stanbio, Boerne, TX) reflectance meter using the Stanbio β-Hydroxybutyrate LiquiColor® assay kit (Stanbio, Boerne, TX). A single dilution was used to expand the readable range to up to 22mM. Before testing the samples, a calibration was performed using 2 lot-specific Ketosite® check cards that have been laboratory tested to yield an accuracy correlation of y = 0.986x + 0.04,with y = Ketosite® (n=114). Once both check cards gave the reading of "CHECK PASS," plasma samples were run. The frozen plasma samples were prepared by bringing them to room temperature while sitting on the bench top for several minutes. KetoSite® Diluent (Stanbio, Boerne, TX) was used to dilute the sample in order to increase the range for the BHB readings. The KetoSite® Diluent was stored in the freezer at – 20 degrees Celsius and then after opening was stored in the refrigerator at 4 degrees Celsius for one month. The KetoSite® Diluent was allowed to rise to room temperature before using. The samples were processed by making a 1/6 dilution by placing 5 µl of plasma sample and 25 µl of KetoSite® Diluent into clean micro-centrifuge tubes, and then gently vortexing the tube to ensure the solution was adequately mixed. The KetoSite® test cards were stored at room

temperature and kept there during testing. The sample testing began with locking a test card into the KetoSite® analyzer. The screen would display "Checking" until the test card was cleared for use at which time the display would ask for the sample. Twenty-two µl of plasma was delivered by a pipette to the reagent pad in the middle of the test card and the door was closed immediately. Each sample testing took 1-2 minutes to process before the raw results would then be displayed. The raw scores were recorded in a laboratory manual and then placed in a MS-Excel spreadsheet and converted to the final reading by accounting for the dilution factor as well as the trace quantity of BHB found in the KetoSite® Diluent. The formula used is as follows: raw data x 6 – 0.01 = mMol/L of sample.

Sacrifice/Tissue collection

The EL mice were sacrificed by decapitation after 4.5 – 5.5 weeks on study. Whole brains, hearts, kidneys, livers, and fat were obtained for possible analysis at a later date. The common area in the animal lab was divided into 3 stations for the sacrifice. All three stations were thoroughly cleaned with quaternary ammonia. The first station was designated as the blood collection and decapitation station. Mice were taken out of the housing cubby one mouse cage at a time to minimize stress of remaining animals. Once the mouse's cage was placed at the first station, the mouse was picked up by the tail was then securely held. The ear mark was quickly read to verify the identity of the mouse. The

mouse number was then spoken aloud for all laboratory assistants to verify that the materials used for tissue storage were ready for this particular mouse, all marked with this particular mouse's number and treatment group. A blood sample was taken from the submandibular vein as described in the Blood Collection section. Immediately after the blood sample was taken, the mouse was placed in a Decapicone disposable mouse restrainer (Braintree Scientific, Braintree, MA) to securely hold the mouse during the decapitation. The mouse was quickly placed in the mouse restrainer and then placed in a small animal guillotine and the blade was quickly pulled down to allow for the mouse head to be completed separated from the mouse body. The mouse head was taken to a second station for brain extraction. All surgical instruments used during the tissue extraction were soaked in either RNA Later (Fisher Scientific, Atlanta, GA) or another RNase inhibitor as to better preserve the tissue in the event that RNA analysis is conducted in the future. The brain was quickly extracted from the skull using stainless steel scissors and spatulas. The brain tissue was then weighed and placed in aluminum foil and flash frozen in liquid nitrogen. Typically, brains were placed into the liquid nitrogen in less than one minute from the time of sacrifice. The mouse body was taken to the third work station in which the mouse was opened and the liver was quickly perfused with a sterile 0.9% sodium chloride solution until the liver had a visually apparent change in color from a dark red to a pale pink which indicates a significant amount of blood had been perfused out of the liver. The liver, heart, kidney and fat pad were each

removed, weighed, and flash frozen into liquid nitrogen. All surgical instruments were cleaned with soapy water and rinsed with clear water after each sacrifice.

The sterile sodium chloride solution was made by adding 9 grams of sodium chloride to 1 liter of distilled water. The solution and container was then autoclaved for 30 minutes. After cooling the solution was placed in the refrigerator in the PI's bench laboratory, which is maintained at 4 degrees Celsius.

Storage

Upon collection, tissue was quickly placed in aluminum foil and flash frozen by submerging into liquid nitrogen. Tissue was then sealed into plastic bags, separated by tissue (heart, kidney, etc.) and group (C, CCR, KD, or KDCR). Tissue bags were placed in a cardboard box and marked with PI's name and stored in a deep freeze at –80 degrees Celsius.

Statistics

Statistical analysis was conducted using SPSS software (originally named for "Statistical Package for the Social Sciences") on the following data sets: weekly seizure scores, mouse body weights, beta-hydroxybutyrate, and plasma glucose. A general linear univariate analysis of variance was used to test the in-between subject effects and a multivariate analysis of variance to measure more than one

data point per subject. Pearson correlations were used to test variable influences on different factors. Independent variable t-tests were used to analyze variables and their relationship to seizures. To find differences between groups, a Least Significant Difference (LSD) post hoc test was utilized. The LSD analysis is equivalent to multiple t tests between all pairs of groups and commonly used for comparison.

Seizure Scoring

Weekly seizure scores were analyzed using a chi-square test with the weekly seizure scores as the dependant variables, the treatment groups (including the control group) as the fixed factor and the change in weight in the 4^{th} week as the covariate. A linear regression with repeated measures was run on the seizure data with the weekly seizure scores as the within-subjects variable and the between-subjects factor was separated (using 0 or 1) by whether or not the mouse was given a ketogenic chow or not (calorie level was not considered with this test). Change in body weight as of the 4^{th} week was used as a covariate in this test. This same linear regression with repeated measures test was ran to determine wither or not a calorie restriction effect was present (with no consideration of ketogenic or not).

The effects change in body weight by week 4 had on seizures were tested using a general univariate analysis of variance. The standard deviation among the data points was calculated using MS-Excel and then the mice were categorized

the mice were categorized into groups: lost weight group (lost more than one standard deviation), no weight change group (within one standard deviation), and gained weight group (gained more than one standard deviation). A univariate analysis of variance was run with the weight categories as fixed factors and the seizure scoring results of week 4 of treatment as the dependent variable. The goal was to examine whether or not these categorical divisions could predict likelihood of seizure control.

Mouse Body Weights

A univariate analysis of variance with the group categories as the fixed variables was run and the change in weight on the 4^{th} week on study was used as the dependent variable. A LSD post hoc analysis was also performed to better determine which groups differed from one another.

Beta-hydroxybutyrate

A univariate analysis was run to analyze plasma ketone levels. The group categories were used as the fixed variables and the plasma levels of beta-hydroxybutyrate levels were used as the dependant variable. This test was run with and without the addition of baseline weight used as a covariate. A LSD post hoc was used in this data analysis when the covariate was not used. This test helps to better determine where differences lay among the groups.

Glucose

A univariate analysis was run to analyze plasma glucose levels. The group categories were used as the fixed variables and the plasma levels of glucose levels at week 4 of treatment were used as the dependant variable. This test was run with and without the addition of baseline weight used as a covariate. A LSD pos hoc was used in this data analysis when the covariate was not used. This test helps to better determine where differences lay among the groups. These same tests were ran with baseline glucose levels to evaluate whether or not levels at treatment week 4 were approaching significance or continuing with homogeneity among groups.

Experimental diets

Composition of Diet

In order to adequately control for diet composition and caloric intake effects, diets were designed to provide consistent amounts of protein, fiber and micronutrients across all groups. This design is especially important as deficiencies of these nutrients have the potential to exert independent effects. Furthermore, our desire was to mimic ketogenic diets used in clinical practice. Typical ketogenic diets designed for humans provide Dietary Reference Intake levels (RDAs or AIs) of these nutrients (via food and supplements) to prevent deficiencies. This standardization of nutrients was accomplished in the calorie restricted groups by formulating purified diets composed of a higher concentration in protein, fiber and

micronutrients per gram, thus requiring less chow to provide the same protein, fiber and micronutrient content as the normocaloric groups. In this study, the fiber source, cellulose, was not calculated in the total carbohydrate amounts.

All diets in this study were designed in cooperation with Melissa Hansen-Petrik, PhD, RD, LDN, of the University of Tennessee Department of Nutrition, Knoxville,TN, and Barbara Mickelson, PhD, of Harlan-Teklad, Indianapolis, IN. The control diet consisted of a standard semi-purified pelleted rodent chow and was fed to the control group *ad libitum*. This control diet delivered 3.8 kcal/g of energy, 90g protein/kg, 696g carbohydrate/kg, 70g fat/kg, 48g fiber/kg. The control calorie restricted diet consisted of a modified semi-purified pelleted rodent chow that delivered 3.8 kcal/g of energy, 121g protein/kg, 619g carbohydrate/kg, 94g fat/kg, and 64g fiber/kg. This pelleted chow was pre-weighed and fed to the mice daily. Both of the ketogenic diets were formed into a paste and consisted of a modified TD96355 (Harlan-Teklad, Madison, WI), which was based on the 4:1 (4g fat: 1g protein + carbohydrate) ratio of the classical KD. The ketogenic diet delivered 6.7 kcal/g of energy, 158g protein/kg, 17g carbohydrate/kg, 662g fat/kg, and 86g fiber/kg. The calorie-restricted ketogenic diet delivered 6.1 kcal/g, 197g protein/kg, 21g carbohydrate/kg, 585g fat/kg, and 105g fiber/kg. The percent for each nutrient is listed in table 2. The ratio of fat to non-fat grams for each group is as follows: 0.09:1 for the control group; 0.13:1 for the control calorie restricted group; 3.77:1 for the ketogenic diet group; and 2.69:1 for the ketogenic calorie restricted group.

Each of these ketogenic diets were pre-weighed and given to the mice daily. The experimental ketogenic diets were designed to match total caloric intake, protein intake, and micronutrient intake of the control group, so *only fat and carbohydrate intake were different*. Calorie-restricted mice (control calorie restricted and ketogenic calorie restricted groups) consumed diets modified and weighed to provide equivalent amounts of protein, fiber, and micronutrients, but with carbohydrate and fat content reduced to achieve 75% of the calorie level consumed by unrestricted control group. Chow amounts were determined by average energy intake of all mice included in the first set to be randomized. Following randomization, experimental diets were fed daily for a period of 4.5-5.5 weeks. Dietary intake for the *ad libitum* fed control group was measured and recorded weekly.

Calculation of Diet Amounts

Before the first set of EL/Suz (EL) mice were randomized, their weekly intake was measured and recorded. These data were used to estimate an average daily intake for the mice of 16.34 kcal per day. This average was used as a baseline caloric intake for mice in all groups. The ketogenic diet group received virtually the same calorie amount with 2.4g of ketogenic diet chow providing 16.08 kcal per day. The two calorie restricted groups received 75% of this computed average. The control calorie restricted groups received 3.2g of the

specially designed pelleted chow which provided 12.16 kcal per day. The ketogenic calorie restricted group received 2.0 grams of the high fat chow providing 12.20 kcal per day. Details of daily diet amounts are summarized in table 3.

Feeding Methods

The researchers were first exposed to the EL mouse model at Boston College

Table 2 – Diet composition

	C		CCR		KD		KDCR	
Nutrient	**g/kg**	**% kcal**	**g/kg**	**% kcal**	**g/kg**	**% kcal**	**g/kg**	**% kcal**
Protein	89.7	9.5	120.8	12.7	158.3	9.5	196.5	12.8
Carbohydrate	695.8	73.8	619.25	65.2	17.25	1	2.15	1.4
Cellulose	48	11.45	64.32	15.23	86.4	11.7	104.64	15.35
Fat	70	16.7	93.5	22.1	662.23	89.5	584.93	85.8
Vit/min mix	41	-	54.66	-	72.28	-	88.07	-

Dietary Composition: Kcals/g: Control 3.8 kcals/g; Control-CR 3.8 kcals/g KD 6.7 kcals/g;; KD-CR 6.1 kcals/g

Table 3 – Nutrients per meal

Daily Nutrient	C (fed *ad lib*,	CCR (3.2g/day)	KD (2.4g/day)	KDCR (2.0g/day)
Protein	0.38571	0.3866	0.3799	0.3930
Carbohydra	2.99194	1.9816	0.0414	0.0043
Cellulose	0.2064	0.2058	0.2074	0.2093
Fat (g/meal)	0.301	0.2992	1.5894	1.1699
Vit/min mix	0.1763	0.1749	0.1735	0.1761
Calories per	16.34	12.16	16.08	12.20

Boston, MA by Dr. Thomas Seyfried. Dr. Seyfried and his graduate student, John Mantis, in the Department of Biology at 514 Higgins Hall, Chestnut Hill, provided training on seizure testing, breeding suggestions, and handling procedures, and suggested methods of feeding a high fat chow (which has a pasty consistency) to this mouse model. The technique used at Boston College included a glass petri dish, trimmed to a side height of approximately 1cm. This petri dish was filled with the high fat paste chow and then placed face down on the wire rack and then a heavy water bottle was placed on top of the dish so that the dish would stay flush against the wire rack lid, thus making the chow available for the mice in the cage as they could access the diet through the wires of the lid. This feeding design became the starting point for designing a new, more efficient technique. It was determined that the BC feeding method had some limitations such as the inability of the mice to get the chow from behind the wires of the wire bar lids. Chow was observed remaining on the outside of the cages where the dish had been as well as remaining in the dish, assuming the mouse/mice was/were unable to access it through the wire bars.

In designing a revised method to feed the mice in the ketogenic groups a feeding dish that could be suspended in the mouse cage, with no wire or any other object obstructing the mouse access to the chow was used in the current study. A polystyrene 60 x 15mm petri dish with snug fitting lid (Fisher Scientific, Atlanta, GA) was used as the feeding bowl. Round magnets were glued to the back of both the petri dish as well as the petri dish lid. The high fat paste chow was

weighed on an Ohaus Scott II gram scale (Carlton Scales, Powell, TN) and placed in the center of the petri dish. The lid was placed on the petri dish, thus providing an efficient storage method. When the petri dish was placed on the wire rack lid of the cage for feeding, the lid was taken off the petri dish and then placed with the magnet facing down, on top of the wire rack lid. The petri dish itself was then placed, magnet up, on the inside of the cage, with the magnets of the dish matched with the magnets of the petri dish lid on the outside of the wire bar lid. This utilized an attraction among the magnets that secured the dish in place, allowing the mouse easy and complete access to the food. See figure 7 below for an overview of this procedure.

The pelleted chow used for the CCR group needed no special feeding device, as it could be placed into the floor of the shoebox cage. The pellets were weighed on the Ohaus Scout II gram scale and pieces of the pellet were chipped off with a clean knife to decrease the weight to the desired amount. Precision for this scale

a. b. c.

Figure 6 – Magnetic feeding dish: Feeding method for high fat chow paste

a. High fat chow is placed in the center of the petri dish to facilitate easy storage. b. Dish is opened and placed on the wire bar lid. c. Magnets hold feeding dishes in place, allowing for easy access for animals.

was to 0.1g. The pellets were then stored in polystyrene tubes until given to each mouse.

Polystyrene tubes and feeding petri dishes were labeled with a non-toxic permanent marker to identify them by mouse number and the experimental group of which they were a part (Example: #10, KD). Both the feeding tubes and the feeding petri dishes were reused after they were washed in soapy water and air-dried.

The experimental diets were stored in a freezer at -20 degrees Celsius in bulk containers clearly labeled with the expiration date issued by the manufacturer of the different chows. These containers were kept in the bench laboratory located at 1215 W Cumberland Ave, Room 342, Knoxville, TN. Individual daily servings were weighed out 3 to 7 days prior to delivery to the mouse. All chow was weighed on an Ohaus Scott II gram scale (Pine Brook, NJ) that has a precision to 0.1g. The scale was calibrated after arriving at the laboratory and before being used to weigh the chow. Before each weighing session, the laboratory bench area was cleaned with diluted Fisherbrand Versa-Clean Liquid Concentrate (Fisher Scientific, Atlanta, GA). Ketogenic diets were weighed directly into the feeding dishes. The control calorie-reduced pellets were weighed in a pre-tared ceramic petri dish and then transferred into the pre-marked tubes.

The feeding petri dishes for each mouse on a high fat chow were then stored in a plastic sealed bag, each mouse having its own bag. For example, mouse #10 may have 7 feeding petri dishes in one plastic bag, thus having 7 days worth of chow meals ready. These plastic bags were marked with the mouse number and treatment group and were then placed on an aluminum baking pan for easy storage. The tubes filled with the pelleted chow for the control-calorie restricted group were also placed in a plastic bag and on the aluminum pan, marked with the mouse number and group. These aluminum baking pans were then transferred to the food cooler in the animal laboratory and stored on a shelf inside the cooler. This cooler was maintained at 4 degrees Celsius.

Timing of Feeding

Mice were fed between 1600 and 1830 nightly. All laboratory assistants were trained extensively before they took on the responsibility of feeding the mice on study. The aluminum pans with the pre-weighed chow were taken out of the animal food cooler and placed on a stainless steel table in close vicinity to Cubicle 8. Then, one at a time, each cage was removed from the cubicle and placed on a nearby stainless steel table. The wire bar lid was removed and the new food was placed. The prior day's petri dishes for the ketogenic fed mice were removed before placing the dish with the fresh chow. Each cage on study had this same procedure. The control fed mice did not need to be fed daily due to the fact that they were fed *ad libitum* and their wire bar lids were filled with

chow. Yet, to control for this low level of stimulation that occurred during the these feeding procedures, the cages of the mice in the control group were handled in the same manner. A cage that housed a mouse in the control group was removed from Cubicle 8 and the lid taken off and then replaced, thus simulating the stimulation that the other mice were exposed to during the feeding process. The dirty feeding petri dishes and tubes were placed into a designated container in the dirty section of the animal laboratory, so they could be later washed and reused. These empty containers were hand washed in soap and water and air dried every 3-7 days.

To assure mice were fed within the hours of 1600 and 1830, laboratory assistants would confirm via telephone by 1730 that the feeding had been completed. If no call was received by that time, attempts were made to call the laboratory assistant responsible for the night's feeding to verify the feeding would be completed. If that laboratory assistant was not reached, a backup plan assured that feeding was completed before 1830 hours. This system was set in place as a double check to assure that the animals on study were properly fed at the same time every night on the study. Laboratory assistants signed a feeding log placed on the door of Cubicle 8. This log included the time and date the chow was delivered to the mice on study.

CHAPTER IV
RESULTS

Tolerance of Diets

The mice readily accepted and consumed each of the four diets, both control and experimental. Although diet taste and texture were not consistent across groups, primarily due to differing macronutrient fat and carbohydrate concentrations, all mice consumed the individual diets with typical interest and eagerness. Other than expected weight loss experienced by mice in the calorie restricted groups, no adverse effects were observed in the mice while they were on the study. This is consistent with previous observations in this mouse strain when fed a macronutrient modified diet (57). The appearance and activity level of the mice were typical across all groups, similar to appearance and activity of the mice prior to randomization and the control group during the study. This observation was casual and not analyzed, as this study did not utilize specific tools to measure appearance and activity changes among groups.

Influence of Diets on Weight

At baseline, mice had a mean body weight of 23.8g±SEM and no significant differences in baseline body weight were detected among groups by univariate analysis. The two calorie-restricted groups lost weight over the course of the 4-

week study. At week 4, univariate analyses revealed a significant reduction in weight in the control calorie-restricted group ($p<0.01$) and the ketogenic calorie-restricted group ($p<0.05$) from average baseline weights. Assignment to ketogenic diet treatment (KD or KDCR) had no independent effect on weight change from baseline ($p=0.238$). Figure 6 illustrates the weights as they separated during the course of the study.

Influence of Diets on Plasma Beta-hydroxybutyrate Levels

Pre-randomization data analyses showed no significant differences in beta-hydroxybutyrate (BHB) levels among the mice. It was not until the fourth week of treatment that differences among the groups became apparent.

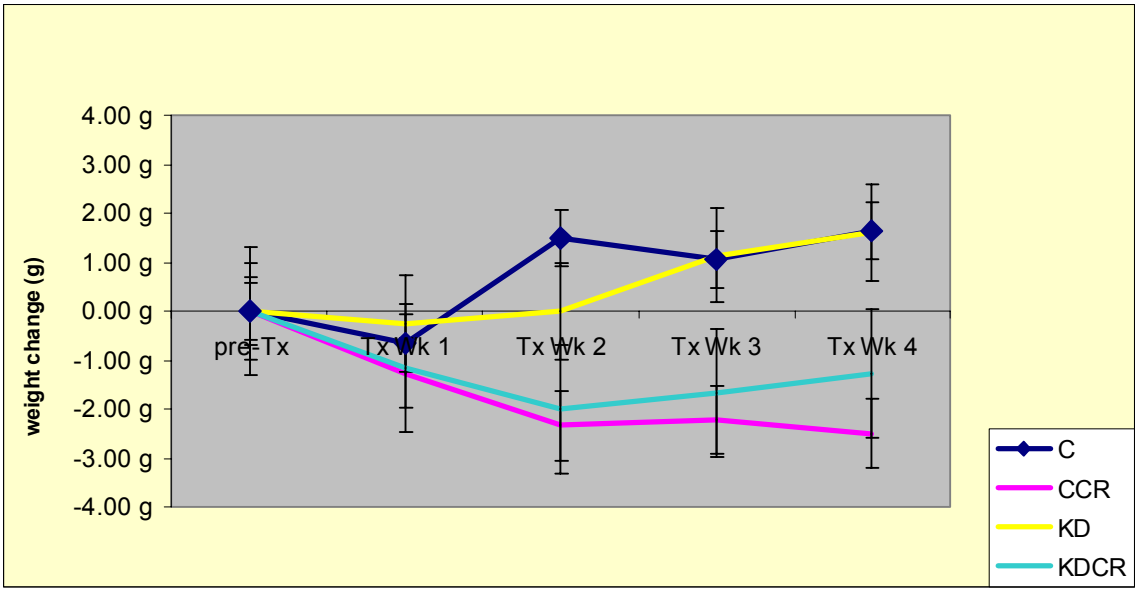

Figure 6 – Change in weight from baseline for each group, before treatment and each week during the treatment. Values shown are means ± SEM.

Results demonstrated a significant difference in BHB levels between treatment groups with baseline weight as a covariate (p=0.029) at week 4. The two groups fed ketogenic diets showed significantly higher BHB levels compared to the control group at the fourth week, as shown in Figure 7. The control calorie-restricted group did not significantly differ from any of the other three groups. Calculated mean levels for groups at week 4 included 0.23± 0.05 mMol/L for the control group (n=5), 0.43±0.05 mMol/L for the control calorie-restricted (CCR) group (n=3), 0.71±0.19 mMol/L for the ketogenic diet (KD) group (n=4), and 0.75±0.19 mMol/L for the ketogenic calorie-restricted (KDCR) group (n=3). Difficulties in blood collection resulted in inadequate volume from some mice and inability to complete all assays.

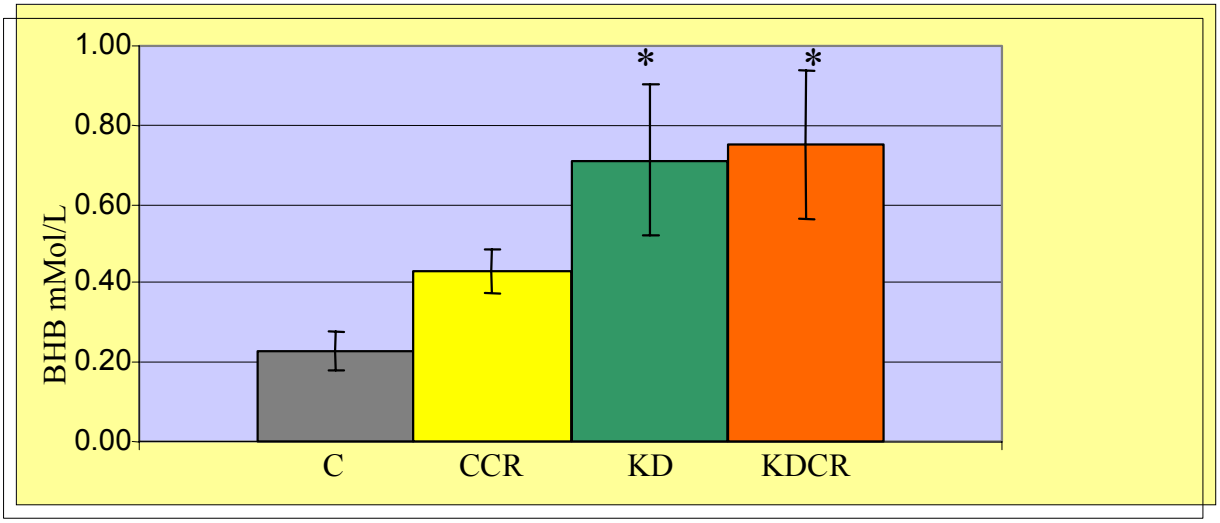

Figure 7 – Plasma beta-hydroxybutyrate levels at week 4 of treatment in EL/Suz mice. Mice on ketogenic diet regimens (KD & KDCR) had significantly higher BHB levels as compared to mice in the control group (C) (p=0.024, p=0.033, respectively). Calorie restriction alone (CCR) did not have a significant effect on plasma BHB levels. Values shown are means +/- SEM.

The control calorie-restricted group did not significantly differ from any of the other three groups.

Influence of Diets on Plasma Glucose Levels

No statistically significant differences in plasma glucose levels were observed among groups at baseline or during the course of the study. Figure 8 shows glucose levels in the control calorie-restricted group approaching significance as compared to the control (p=0.064).

The calculated mean levels for groups at week 4 which included 145±15 mg/dl for the control group (n=4), 94±8 mg/dl for the control calorie-restricted (CCR)

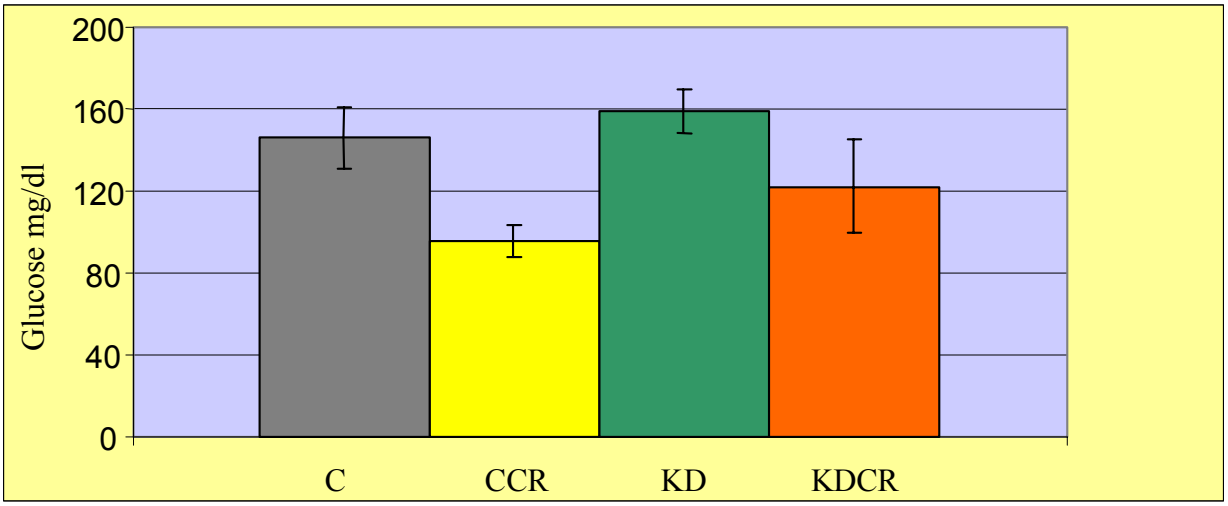

Figure 8 - Plasma glucose levels at treatment Week 4 in EL/Suz mice. No significant differences in glucose levels were observed among groups, although levels in the control calorie-restricted group (CCR) were nearly significantly lower than those of thecontrol group (C) (p=0.064). Values shown are means +/- SEM.

group (n=5), 159±11 mg/dl for the ketogenic diet (KD) group (n=4), and 122±23 mg/dl for the ketogenic calorie-restricted (KDCR) group (n=3).

When the data were analyzed for a main effect of calorie-restriction (C and KD vs. CCR and KDCR), a significant difference was found (p=0.011) with calorie-restricted mice having significantly lower glucose (figure 9). Confirming this finding, a Pearson's correlation showed a significant relationship between weight change and glucose levels by week 4 (*R*=0.568, p=0.017, data not shown) indicating that mice experiencing weight loss were more likely to have lower glucose levels.

Thus, calorie restriction and weight loss, regardless of diet composition, were associated with lower glucose levels.

Figure 9 - Glucose levels at treatment week 4 in calorie restricted mice (CCR and KDCR) exhibited significantly lower blood glucose levels as compared to unrestricted mice (C and KD) (p=0.011). Values shown are means +/- SEM.

Influence of Diets on Seizure Control

No significant differences in seizure control were observed among treatment groups in this study, although possible trends are apparent. Figure 10 graphically displays a trend towards seizure control for the ketogenic calorie-restricted group over the other groups, but no significant differences were detected. Eighty percent of mice in that group had no seizures at week four. In the ketogenic and control calorie-restricted groups, 60% of mice were seizure free, while 40% of the control group was seizure free by week 4 of treatment.

The ketogenic diet groups (KD and KDCR) experienced a trend toward seizure control over the non-ketogenic groups (C and CR) although the differences were not significant. There was also no main effect of calorie restriction when

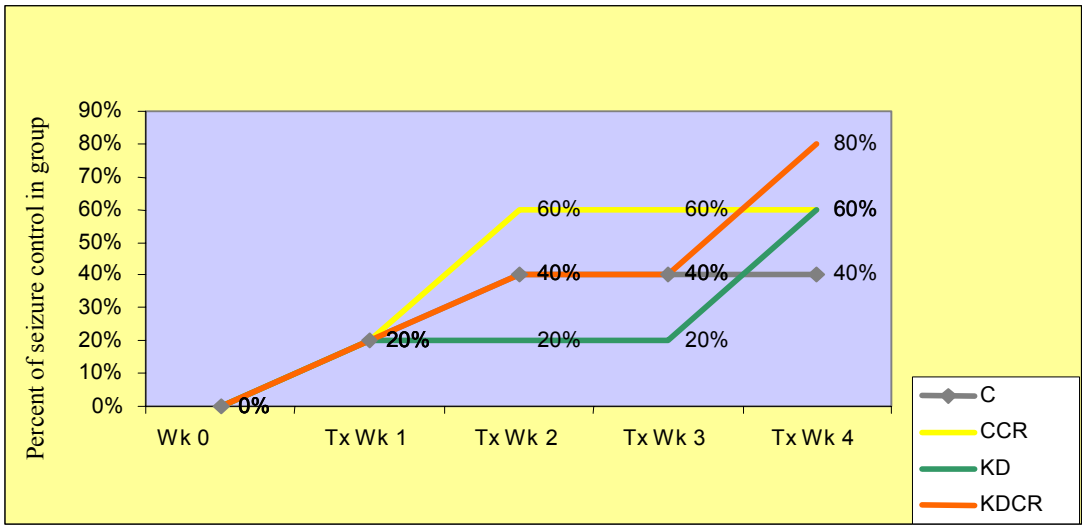

Figure 10 – Percentage of EL/Suz mice in each treatment group seizure free pre and post randomization to diet treatment group. By week 4, seizure control was 40% in the control group (C), 60% in the control calorie-restricted group (CCR), 60% in the ketogenic diet group (KD), and 80% in the ketogenic calorie-restricted group (KDCR).

comparing the calorie-restricted groups (CCR and KDCR) with the non-calorie restricted groups (C and KD).

Weight and biochemical changes according to seizure status

As trends in seizure status were apparent among groups, but not readily detectable likely due to sample size limitations, body weight change, BHB, and glucose values were also analyzed by grouping mice according to seizure status at week 4 (see table 4) to determine the relationship between these outcome variables with seizure status.

Mouse body weight at week 4 was significantly related to seizure control

Table 4 – Weight and Biochemical changes at week 4 of treatment

	Seizures (n=8)	No Seizures (n=12)
Body weight change baseline to week 4 (g)	1.58±0.96*	-1.27±0.58*
BHB (mMol/L)	0.31±0.07	0.58±0.11
Glucose (mg/dl)	150±18	125±10

Body weight change, BHB, and glucose levels according to seizure status at week 4. Values shown are means ± SEM. Values designated "*" are significantly different at $p<0.05$. BHB levels approached a near significant difference according to seizure status at $p=0.051$.

(p=0.006). Body weight change from baseline to week 4 was also significantly different in mice that experienced a seizure at week 4 compared to mice that did not (p=0.015). Mice that lost weight by week 4 were more likely to be seizure free, independent of treatment group.

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Seizure free mice had higher BHB levels than did mice with seizures at week 4, although this did not reach statistical significance (p=0.051). No relationship between glucose levels and seizure control was observed (p=0.230).

CHAPTER V
CONCLUSIONS AND DISCUSSION

It has been clearly established that the ketogenic diet is effective in controlling seizures (25, 46, 47) . Although the treatment was stumbled upon many years ago, even today, *how* the diet works remains unclear. Researchers from all over the world (40) seek to find those answers and many theories have been proposed.

When investigating efficacy of a dietary treatment in an animal model, experimental diet design is of utmost importance. In this case, experimental ketogenic diets designed to test mechanisms must be tightly controlled and reflect the composition of the clinical diet, as efficacy has been shown time and time again (25, 46, 47). Prior animal research has largely ignored this mantra (57) and this limits applicability of findings from those studies.

Previous studies of ketogenic diet efficacy in animal models of epilepsy have not controlled for protein, fiber, and micronutrients (57). Rather, diet manipulation has focused on carbohydrate and fat differences and energy restriction without regard for other dietary components or utilization of a proper control diet. When displacing macronutrients or when restricting calories, typical study designs simply change amounts of fat and carbohydrate or simply limit the amount of food provided (57). When restricting portions, all nutrients are affected; in

addition to reducing total energy intake, animals also consume a protein, fiber, and micronutrient deficient diet. Energy restriction implemented in this way can influence metabolism and alter gene expression. For example, low protein intake has been shown to be affect brain function (77). Therefore, it is unclear whether findings from such studies are due solely to energy restriction or if results are also influenced by protein deficiency, altered fiber intake, or micronutrient deficiency. Fiber can impact absorption, which directly affects metabolism (79, 81). Vitamins and minerals are utilized in many metabolic pathways (83), therefore availability of these nutrients in experimental diets needs to be standardized when seeking mechanisms respondent to diet manipulation.

Therefore, the aim of the present study was to test the efficacy of a novel highly-controlled experimental diet design as a new gold standard for ketogenic diet manipulation to search for mechanisms in animal models of epilepsy. This study is innovative, as nutrient composition was strictly controlled across groups for protein, fiber, and macronutrient intake while manipulating only the energy intake and content of carbohydrate and fat.

The results from this study suggest that ketosis indicated by elevated BHB levels and energy restriction indicated by weight loss correspond to lower seizure susceptibility in an experimental model of naturally occurring epilepsy. This is consistent with previous findings (57) but is even more telling because dietary components, excepting carbohydrate and fat, were consistent across groups and

the overall diets reflected the ketogenic diet in clinical practice rather than an extreme not applicable to humans. While there was an apparent trend, a statistically significant difference in seizure control was not observed among individual experimental diet groups. It is likely that a non-extreme diet intervention, as used here, requires a larger sample size in order to detect differences among groups. Thus, a larger sample size could have potentially better delineated these trends and more clearly defined the effects.

Importantly, the observation that KD fed mice with or without energy restriction had higher BHB levels and mice without seizures at week 4 had a nearly significantly higher serum BHB level is consistent with prior reports that ketogenic diet treatment reduces seizure susceptibility in EL mice (57). It is possible that BHB plays a direct role in seizure control as has been previously observed (56). It is also possible that BHB is only serving as a marker of metabolic changes consistent with ketogenic diet treatment as has been suggested by others (86). The observed connection between weight loss and seizure susceptibility is likewise consistent with the profound effect of energy restriction observed previously by Mantis *et al.* (57). They found that a reduced caloric intake, regardless of the source of the kcalories, was more anti-epileptic than a ketogenic diet (57). It is possible that the implementation of energy restriction in these studies concluded efficacy due to the more severe nature of the energy restriction itself or the deficiency of protein or other micronutrients. Mantis and his group induced a weight loss of 20-23% of baseline body weight. Regardless

of caloric sources, this could be construed as *truly starving* the mouse as opposed to mimicking the metabolism of starvation, such as with a ketogenic diet. This type of starvation is not clinically applicable. The small sample size of the present study limited the ability to detect a potential direct effect of energy restriction on seizure control. For example, one mouse in the calorie-restricted ketogenic diet group gained weight. As mice in each group were provided an identical quantity of food regardless of starting weight it is possible that some energy-restricted mice received quantities greater than their physiological requirement. It is likewise possible that some non-calorie restricted mice received less than their physiological requirement. Therefore, possibly the energy restriction of some mice could have caused unexpected seizure control. In all, a larger sample size could have strengthened the ability to detect a direct effect of energy restriction. Consequently, it is helpful to examine the effect of energy restriction by using weight change as a proxy. When seizure susceptibility was analyzed, weight did prove to be a strong predictor of seizure control, with improved seizure control in mice with lower body weights and/or weight loss from baseline. Certainly, weight loss could be considered a proxy for energy restriction whereas weight gain could be considered a proxy for energy excess. Interestingly, a ketogenic effect on weight was not observed.

In this study there appeared to be an intermediate, albeit non-significant, effect on BHB with energy restriction alone, which might be expected. Interestingly, Mantis *et al*. found BHB levels increased in the control calorie-restricted group as well as both ketogenic diet groups (57). This inconsistency from the current

findings is likely due to the extreme calorie restriction Mantis and his team placed on the calorie-restricted groups more clearly eliciting ketosis.

Calorie-restricted mice (fed either ketogenic or control diets) did have significantly lower glucose levels than the mice that were fed ketogenic diets ($p=0.011$). Mantis observed a correlation between lower blood glucose levels and seizure control in EL mice (57) and postulated that adequate glucose levels are necessary to precipitate a seizure. Currently, researchers at Massachusetts General Hospital are exploring the connection between reducing serum glucose and seizure control clinically by placing children on a low glycemic index treatment (LGIT) in order to maintain lower glucose levels. This diet is reported to be easier on families to administer and less restrictive. Some preliminary data on this deviation of the classic ketogenic diet appear promising (44), yet clinicians studying this diet admit it is doubtful that the LGIT will replace or be as effective as the classic ketogenic diet (Dr. EA Thiele, Massachusetts General, live presentation, Boston, MA, 2006). This coincides with one of the current hypotheses that ketogenic diets could be effective due to limited glucose availability for precipitation of a seizure (54, 55).

Uncovering the mechanism(s) of action for the ketogenic diet is a much-needed effort, which could ultimately be used to refine the diet treatment, thereby making it more effective. Also, understanding more about the diet could expand its use and improve more lives affected by epilepsy. If the diet could be simplified, it

would likely be more aggressively used for intractable epilepsy or possibly be used earlier in treatment to avoid undesirable side effects of anti-epileptic drugs. Further, uncovering the mechanism(s) of action could give great insights into the disorder of epilepsy. Experiments utilizing laboratory animals can lead to better understanding the way in which ketogenic diets alter substrate utilization in the brain could give insight into *how* this diet can control seizures. Processes and tissue utilization can be explored and then applied to humans. Understanding what effectively *treats* a disorder, could allow better understanding of the disorder itself. Yet, researchers have not stopped at epilepsy, but are now questioning whether ketogenic diets have potential roles in other disease states. Researchers have reported some positive preliminary results in treating Parkinson's disease (87, 88) , amyotrophic lateral sclerosis (ALS) (89), traumatic brain injury (87), stroke (87), Alzheimer's (87), obesity with high cholesterol (90), prostate cancer (91, 92) and brain cancer (93-95) with ketogenic diets.

Perhaps Macfadden, the fanatical fitness guru of the early 1900s, wasn't as "crazy" as researchers may have presumed when he promoted his ideas of curing all disease states by fasting and exercise (13). Although it is unlikely that all disease states can be treated with a diet that mimics fasting, it does appear as though researchers have only scratched the surface of the potential roles ketogenic diets may play in healthcare in the future. Yet, before all the metabolic alterations induced by this treatment in the human body can be fully understood, researchers must first look at how animal models are influenced by

this treatment. And since this treatment is a therapeutic *diet*, the design of experimental diets to study it must be well controlled if these studies are to give meaningful insight into the mechanisms of action. The experimental diet design utilized in the present study is the first tightly controlled ketogenic diet design to be described and, if adopted by basic scientists in the ketogenic diet research community, has the potential to make research results in this area more meaningful and applicable to the human population.

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